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16. ABSTRACT

This report summarizes research results and progress in the following general areas concerned with chemistry, metabolism and mode of action of organophosphorus, carbamate, and organochlorine insecticides. Contents:

A. Insecticide Metabolism and Mode of Action
B. Insecticide Selectivity
C. Structure-Activity Relationships
D. Biological and Environmental-Alteration of Insecticides
E. Effect of Impurities on the Potentiation of Organophosphorus Insecticides
F. Delayed Toxicity of Trialkyl Phosphorothioates
G. Delayed Neurotoxicity of Organophosphorus Esters
H. Mode of Action of Phosphoramidothioate Esters
I. Gas Chromatography of Insecticides
J. Neurophysiological Studies on the Mode of Action of Insecticides

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CHEMISTRY AND MODE OF ACTION OF INSECTICIDES

Phase II

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ABSTRACT

This report summarizes research results conducted under EPA Research Grant No. R-804345 for the period February 15, 1976 - May 31, 1981. Progress in the following general areas concerned with the chemistry, metabolism and mode of action of organophosphorus, carbamate, and organochlorine insecticides is reported.

A. Insecticide Metabolism and Mode of Action

B. Insecticide Selectivity

C. Structure-Activity Relationships

D. Biological and Environmental-Alteration of Insecticides

E. Effect of Impurities on the Potentiation of Organophosphorus Insecticides

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A. INSECTICIDE METABOLISM AND MODE OF ACTION

1. Studies on the Oxidative Conversion of P=S to P=O Esters and the Effect of Chirality on Toxicological Properties.

Introduction - Metabolic oxidation reactions are of considerable significance in the *in vivo* modification of insecticides in plants and animals. The various oxidation reactions which take place, e.g.,olefin epoxidation, thioether oxidation to sulfoxides and sulfones, *N*- or *O*-dealkylation, aliphatic and aromatic hydroxylation, and phosphorothionate desulfuration (P=S to P=O conversion), result in both activated and detoxified products and are mediated by the mixed-function oxidases (MFO).

In the pure state, phosphorothionate esters such as parathion are poor inhibitors of the cholinesterase enzymes owing to the deactivating influence of the thionate sulfur atom on the reactivity of the phosphorus center. By contrast, the corresponding phosphate ester, paraoxon, is a very potent anticholinesterase, and the toxic action of parathion and related insecticides is attributable to the phosphate ester formed *in vivo* by oxidative desulfuration. In order to elucidate the mechanism of the desulfuration reaction, we have examined the stereochemistry of P=S to P=O conversion effected by chemical model oxidation systems and mouse liver MFO, using the resolved isomers of fonofos (*O*-ethyl *S*-phenyl ethylphosphonodithioate). This report is concerned with the stereochemical aspects of the chemistry, metabolism, and mode of action of the chiral isomers of fonofos.

Synthesis and Determination of Absolute Configuration - The various reactions used in the synthesis of the chiral isomers of fonofos relevant to this study are indicated in Scheme 1 for one of the enantiomers. Values of observed (neat) and specific rotations of all enantiomeric materials are presented in Table 1. The absolute configuration of the resolved starting material, i.e., enantiomeric *O*-ethyl ethylphosphonothioic acid (1a or 1b), was established by x-ray diffraction analysis of the (-)-α-phenethylammonium salt of the acid (2a), obtained after repeated crystallizations. The absolute configuration of the acid 1a was established as (*S*)$_P$ by relating it to the known configuration of the (*S*)-(-)-α-phenethylammonium ion. Since the phosphonothioic acid 1a is (*S*)$_P$, its enantiomer 1b is (*R*)$_P$.

Treatment of 1a or 1b with phosphorus pentachloride in carbon tetrachloride afforded (-)- and (+)-*O*-ethyl ethylphosphonochloridothioate, respectively. The reaction probably proceeded with a small amount of racemization since treatment of the chloridothioate 3a with sodium hydroxide in aqueous dioxane resulted in 1a with 7-15% reduction in optical rotation.

SCHEME 1

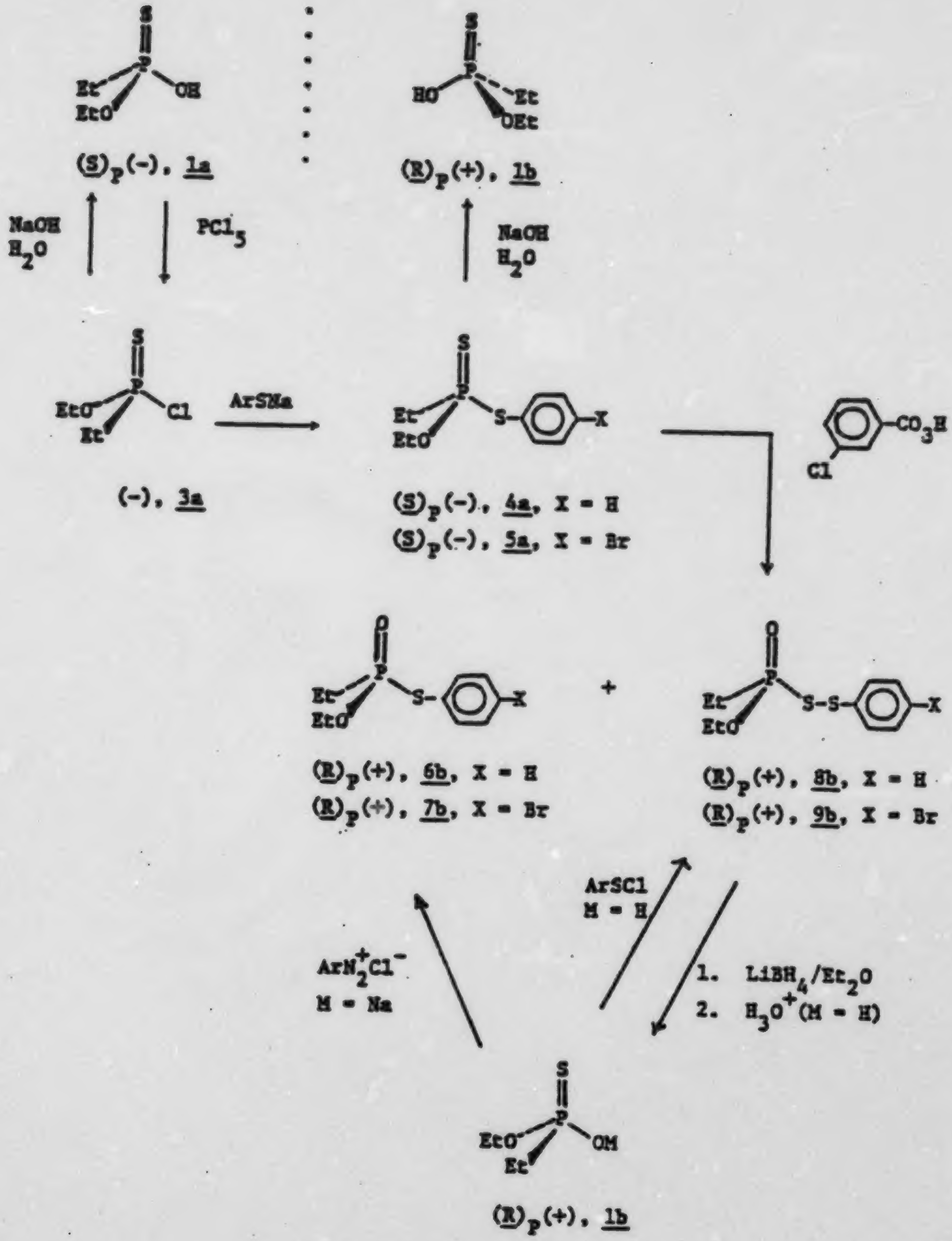
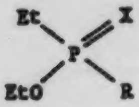
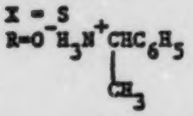

(S)$_P$(−), 1a
(R)$_P$(+), 1b
NaOH H$_2$O
PCl$_5$
NaOH H$_2$O
ArSNa
(−), 3a
(S)$_P$(−), 4a, X = H
(S)$_P$(−), 5a, X = Br
(R)$_P$(+), 6b, X = H
(R)$_P$(+), 7b, X = Br
(R)$_P$(+), 8b, X = H
(R)$_P$(+), 9b, X = Br
ArSCl M = H
ArN$_2^+$Cl$^-$ M = Na
1. LiBH$_4$/Et$_2$O
2. H$_3$O$^+$(M = H)
(R)$_P$(+), 1b

TABLE 1. PHYSICAL CONSTANTS AND VALUES OF OBSERVED (NEAT) AND SPECIFIC ROTATIONS IN DEGREES ARC FOR THE COMPOUNDS OF CENTRAL STRUCTURE



Structure	No.	Absolute configuration	Observed α_D^{24} (neat)	Specific[a] $[\alpha]_D^{24}$
X = S	1	Racemic	-	-
R = OH	1a	$(S)_P$	-15.45	-
	1b	$(R)_P$	+14.82	-
X = S	2a	$(S)_P$	-	-12.78[b]
 $R=O^{-}H_3N^{+}CH(CH_3)C_6H_5$	2b	$(R)_P$	-	+12.40[b]
X = S	3	Racemic	-	-
R = Cl	3a	-	-82.50	-
	3b	-	+78.65	-
X = S	4	Racemic	-	-
R = SC_6H_5	4a	$(S)_P$	-181.50	+138.40[c]
	4b	$(R)_P$	+155.98	-114.56
X = S	5	Racemic	-	-
R=SC_6H_4-p-Br	5a	$(S)_P$	-257.90[d]	+130.67[c]
	5b	$(R)_P$	+258.13	-129.53
X = O	6	Racemic	-	-
R = SC_6H_5	6a	$(S)_P$	-	-121.70
	6b	$(R)_P$	-	+112.6
X = O	7	Racemic	-	-
R=SC_6H_4-p-Br	7a	$(S)_P$	-	-107.87
X = O	8	Racemic	-	-
R = SSC_6H_5	8a	$(S)_P$	-32.30	-68.27
X = O	9	Racemic	-	-
R=SSC_6H_4-p-Br	9a	$(S)_P$	-65.50	-138.38[e]

Note a. In cyclohexane solvent unless specified.

Note b. In methanol solvent.

Note c. A change in sign of rotation from the neat measurements also was observed in other solvents.

Note d. Rotation of melted material was measured.

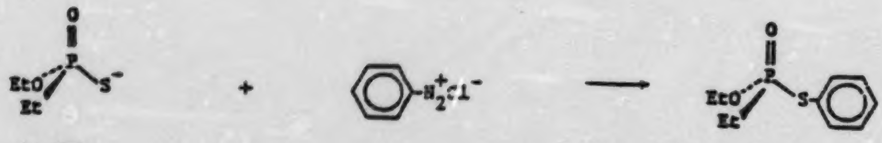
Note e. In carbon tetrachloride solvent.

Reaction between the enantiomeric O-ethyl ethylphosphonochloridothioate (3a and 3b) and the sodium salt of benzenethiol or p-bromobenzenethiol resulted in the corresponding enantiomers of fonofos (4a and 4b) and the p-bromo analogs (5a and 5b). The absolute configuration of the (-)-rotating isomer 5a [α_D^{24} -257.90° (neat after melting)] was determined by x-ray analysis and established as (S)p. Since 5a is (S)p, 5b is (R)p.

The configurations of the fonofos enantiomers (4a and 4b) were assigned on the basis of their chemical similarity to the p-bromo analogs (5a and 5b). For example, both 4a and 5a were prepared from (-)-O-ethyl ethylphosphonochloridothioate, which in turn was synthesized from (S)p(-)-O-ethyl ethylphosphonothioic acid (1a). Further, alkaline hydrolysis of 4a and 5a both returned (R)p(+)-O-ethyl ethylphosphonothioic acid (1b), the expected isomer from three inversion reactions. Rotations (neat, degrees arc) for the various products in the three-reaction cycle involving the preparation and alkaline hydrolysis of fonofos (4a) and the p-bromo analog (5a) are given as follows:

α_D^{24}	1a -14.50°	3a -77.71°	4a -149.20°	1b +11.43°
α_D^{24}	1a -14.94°	3a -82.50°	5a -257.99°	1b +14.65°

Based on the rotations of the starting acid 1a and the recovered acid 1b, the three-reaction cycle involving fonofos proceeded with about 21% racemization. The reaction cycle was significantly cleaner with the p-bromo analog where only 2% racemization was observed. The enantiomeric oxons of fonofos (6a and 6b) and corresponding p-bromo analog (7a) were prepared by the reaction between the sodium salt of 1a or 1b and the respective aryl diazonium chloride. This reaction undoubtedly proceeds by reaction between the aryl carbonium ion and the anion of 1a or 1b and bonds connected to the phosphorus atom are not involved. Therefore, the configurations of the oxon should be identical to that of the acid. Since the configuration of the acid 1a by x-ray analysis was determined to be (S)p, 6a and 7a are (S)p and 7b is (R)p.



1a (S)p

6a (S)p

The configurations of (-)-phenyl ethyl(ethoxy)phosphinyl disulfide (8a) and the corresponding p-bromo analog (9a) were assigned by two independent methods. The first was based on their synthesis from the appropriate arylsulfenyl chloride and the acid 1a (see Scheme 1). As in the case of the synthesis of the oxons, the chiral phosphorus atom is not involved in this reaction and 8a and 9a should have the same configuration as 1a. Second, lithium borohydride reduction of 8a or 9a, resulting in cleavage of the S-S bond, returned 1a with greater than 99% optical retention in both cases. Observed rotations (neat, degrees arc) or products involved in this reaction cycle are given as follows:

α_D^{24}	1a -14.50°	8a -32.30°	1a -14.40°
α_D^{24}	1a -14.50°	9a -65.50°	1a -14.45°

These reactions show that the configurations of 8a and 9a are identical to 1a and, therefore, they are $(S)_P$.

Oxidation with *m*-Chloroperoxybenzoic Acid - Oxidation of the enantiomers of fonofos (4a and 4b) with m-chloroperoxybenzoic acid in dichloromethane gave fonofos oxon and phenyl ethyl(ethoxy)phosphinyl disulfide as the principal products. Other materials isolated were unreacted fonofos, diphenyl disulfide, and diphenyl disulfide oxide. Data for the specific rotations (cyclohexane) of the oxon and phosphinyl disulfide isolated from oxidation of 4a and 4b are presented in Table 2. From the sign and magnitude of the specific rotations of the oxon and disulfide isolated, it is apparent that (S)p-fonofos (4a) was converted predominantly to (R)p-fonofos oxon (6b) and (R)p-phenyl ethyl(ethoxy)phosphinyl disulfide (8b). Conversely, (R)p-fonofos (4b) was converted primarily to 6a and 8a.

TABLE 2. SPECIFIC ROTATIONS IN CYCLOHEXANE (DEGREES ARC) OF THE CHIRAL PRODUCTS OBTAINED FROM PERACID OXIDATION OF FONOFOS ENANTIOMERS.

Fonofos enantiomer[a]	$[\alpha]_D^{24}$, Peracid Products oxon	disulfide
4a, S_P	+66.17 (c 0.141)	+55.40 (c 2.22)
4a, $(S)_P$	+66.32 (c 0.117)	+54.32 (c 1.81)
4b, $(R)_P$	-57.87 (c 1.30)	-51.86 (c 1.84)

Note a. $[\alpha]_D^{24}$ of synthesized 4a and 4b were +138.20 (c 0.302) and -114.56 (c 0.309) respectively.

An approximation of the stereospecificity of the peroxy acid oxidation of fonofos was made from the specific rotations of the products and an estimate of the optical purity of the enantiomeric fonofos, synthesized oxon and phosphinyl disulfide. Thus, the oxidation of *4a* to *6b* was estimated to occur with about 77% retention of configuration and oxidation of *4a* to *8b* with about 90% inversion.

Toxicological Properties of the Fonofos Chiral Isomers - Toxicity data for the racemic and optically active enantiomers of fonofos and fonofos oxon against susceptible house flies (NAIDM strain), *Culex* mosquito larvae, and white mice are presented in Table 3. Included also are values for the relative toxicity ratio, defined as the LD_{50} or LC_{50} of the racemate or less toxic enantiomer divided by the corresponding values for the more toxic isomer, i.e., the more toxic isomer was given the value of unity. In the case of fonofos, the $(\underline{R})_p$ isomer was more toxic against all test animals than the $(\underline{S})_p$ enantiomer and the racemates were intermediate in toxicity. The difference in toxicities of the two isomers evidently varied with the test animal and largest difference was observed with the house fly (toxicity ratio of 4). In comparison, the toxicity ratio for mosquito larvae was only 1.8. While the toxicity ratios are not large, there is little doubt that chirality of the phosphorus center has a significant effect on the toxicity of the fonofos isomers.

Much larger differences in toxicity were observed between the fonofos oxon isomers and higher toxicity was found with the $(\underline{S})_p$ enantiomer. For example, $(\underline{S})_p$-oxon was approximately 12-fold more toxic to house flies relative to the less toxic $(\underline{R})_p$ enantiomer. The same toxicity ratio was observed with mosquito larvae. By oral administration $(\underline{S})_p$-fonofos oxon was 6-fold more toxic to mice than the $(\underline{R})_p$-oxon but only a 2.6-fold difference was observed after intraperitoneal administration.

Cholinesterase Inhibition - The bimolecular rate constants (k_i) for the inhibition of bovine erythrocyte (BAChE), electric eel (EEAChE), house fly-head (HFAChE) acetylcholinesterases, horse serum (HSChE) and cholinesterases from different mouse tissues are presented in Table 4.

Relative values for k_i, i.e., where the anticholinesterase activity of the less active $(\underline{R})_p$-fonofos oxon isomer is given the value of unity, are presented parenthetically. These values or k_i ratios provide a measure of the relative inhibitory potency of the oxon isomers. For example, the k_i ratio given for the inhibition of HFAChE by $(\underline{S})_p$-oxon indicates that this isomer is 62.5-fold more potent as an anticholinesterase than the $(\underline{R})_p$ isomer.

The data reveal that variable but significant rate differences exist in the inhibition of ChE by the enantiomers of fonofos oxon against the different ChE enzymes. In every case, $(\underline{S})_p$-fonofos oxon was a more potent inhibitor than the $(\underline{R})_p$ enantiomer. In general, the $(\underline{S})_p$-oxon was approximately 2-fold more effective as an inhibitor than the racemic mixture. The largest difference in anticholinesterase activity between the two

TABLE 3. TOXICITY OF THE ENANTIOMERS AND RACEMATES OF FONOFOS AND FONOFOS OXON AGAINST HOUSE FLIES, MOSQUITO LARVAE AND WHITE MICE.

Compound	House fly		Mosquito larvae		Mouse (oral)		Mouse (i.p.)	
	LD_{50} (µg/g)	Ratio	LC_{50} (ppb)	Ratio	LD_{50} (mg/kg)	Ratio	LD_{50} (mg/kg)	Ratio
Fonofos racemic	12.0	1.9	31.0	1.2	14.0	1.3	4.8	1.3
$(\underline{S})_P$	25.0	4.0	45.0	1.8	32.0	3.4	7.5	2.0
$(\underline{R})_P$	6.3	1.0	25.0	1.0	9.5	1.0	3.8	1.0
Fonofos oxon racemic	8.0	2.0	92.0	1.8	21.0	3.5	0.94	1.3
$(\underline{S})_P$	4.0	1.0	50.0	1.0	6.0	1.0	0.72	1.0
$(\underline{R})_P$	48.0	12.0	610.0	12.2	38.0	6.3	1.86	2.6

TABLE 4. VALUES FOR THE BIMOLECULAR RATE CONSTANT FOR THE INHIBITION OF DIFFERENT CHOLINESTERASE ENZYMES BY RACEMIC AND ENANTIOMERIC FONOFOS OXON.

Enzyme	$k_i \times 10^{-5}$ ($\underline{M}^{-1}min^{-1}$)		
	racemic	$(\underline{S})_P$	$(\underline{R})_P$
1. Electric eel AChE	28 (3.3)[a]	78 (9.3)	8.4 (1)
2. Bovine erythrocyte AChE	16 (4.6)	32 (9.0)	3.5 (1)
3. Fly-head AChE	130 (14.8)	550 (62.5)	8.8 (1)
4. Bee-head AChE	-	12 (10.9)	1.1 (1)
5. Horse serum ChE (ASCh)[b]	7.5 (2.5)	13 (4.3)	3.0 (1)
(BuSCh)[c]	6.2 (1.9)	13 (4.0)	3.2 (1)
6. Mouse brain ChE	26.0 (26)	49 (49.0)	1.0 (1)
7. Mouse whole-blood ChE	31.0 (22)	85 (56.7)	1.4 (1)
8. Mouse serum ChE (ASCh)	1.4 (4.7)	3.7 (13.2)	0.3 (1)
(BuSCh)	1.7 (3.4)	4.1 (5.3)	0.8 (1)
9. Mouse liver ChE	-	4.4 (10.5)	0.4 (1)

Note a. Values in parentheses represent relative rate constants.
Note b. Acetylthiocholine was used as substrate.
Note c. Butyrylthiocholine was used as substrate.

enantiomers was found in the inhibition of HFAChE (62.5-fold), followed by mouse whole-blood (56.7-fold) and brain (49-fold) ChE. Further, with a k_i value of 5.5 X 10^7 $\underline{M}^{-1}min^{-1}$ for the (S)p isomer, HFAChE evidently was most sensitive to inhibition among the various enzymes examined. Much smaller differences in k_i values were observed between the enantiomers for the inhibition of serum ChE (no. 5 and 8 in Table 4). Compared to HFAChE, the serum cholinesterases were substantially less sensitive to inhibition by the (S)p-oxon and this was the principal cause for the smaller k_i differences between the (S)p and (R)p isomers. Differences in k_i values between the isomers for the inhibition of BAChE and EEAChE were intermediate to those for HFAChE and serum ChE. Overall, the order of differences in k_i between the isomers were HFAChE > BAChE > HSChE.

The rate differences observed in enzyme inhibition were qualitatively consistent with the differences observed in toxicity between the isomers, i.e., the stronger ChE inhibitors were more potent toxicants. Comparison of house fly toxicity in Table 3 with HFAChE inhibition data in Table 4 shows a 12-fold difference in toxicity between (S)p- and (R)p-fonofos oxon but a 62.5-fold difference in anticholinesterase activity. Similar relationships also were found for mouse toxicity and mouse brain or blood ChE inhibition, e.g., a 6-fold difference in mouse oral toxicity (2.6-fold intraperitoneally) was observed between the (S)p- and (R)p-fonofos oxons compared to 50- and 60-fold differences in the inhibition of the mouse brain and mouse blood cholinesterases. The smaller difference in toxicity between the enantiomers compared to inhibition rates suggests that other processes involved in intoxication and detoxication, particularly the latter, also are affected by chirality.

In Vivo Inhibition of Cholinesterases - The in vivo inhibition of ChE in white mice and house flies after treatment with the enantiomers and racemates of fonofos and fonofos oxon was examined. Figures 1 and 2 provide plots showing enzymatic activity after different time intervals of brain and whole-blood ChE obtained from mice treated with 3.0 and 0.6 mg/kg of the enantiomers and racemates of fonofos and fonofos oxon, respectively. The results reveal a significant difference in the inhibition of brain and whole-blood ChE from mice treated with the two fonofos enantiomers. During the initial 2-4 hr after treatment, both brain and blood ChE were inhibited in mice treated with (R)p-fonofos at a faster rate and to a greater extent than the (S)p isomer. As expected, the rate of inhibition by racemic fonofos was in between that found for the enantiomers. At the common time interval of 2 hr after treatment, more than 80% of the brain and blood ChE obtained from mice treated with (R)p-fonofos was inhibited, compared to 15 and 60% inhibition of the respective enzymes obtained from mice treated with the (S)p isomer. The initial relative rates of inhibition, therefore, are in good agreement with the differences in toxicity between the enantiomers of fonofos (see Table 3).

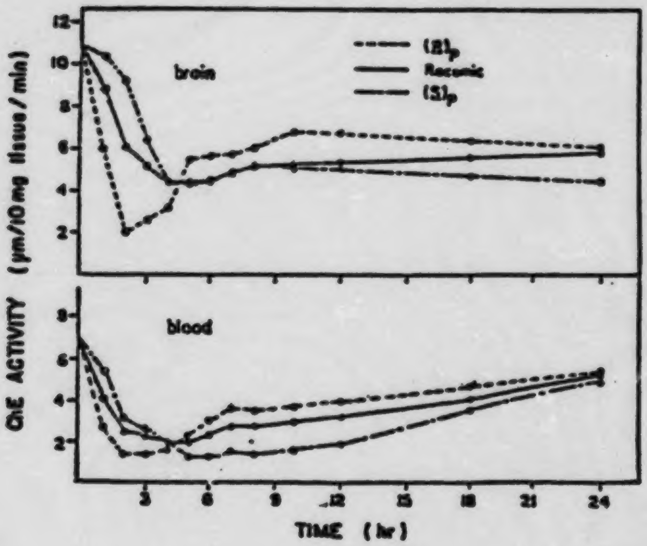


FIG. 1. Level of cholinesterase activity in mouse brain and blood at various time intervals after intraperitoneal administration of 3 mg/kg racemic and enantiomeric fonofos. Each point represents the mean of 3 replications.

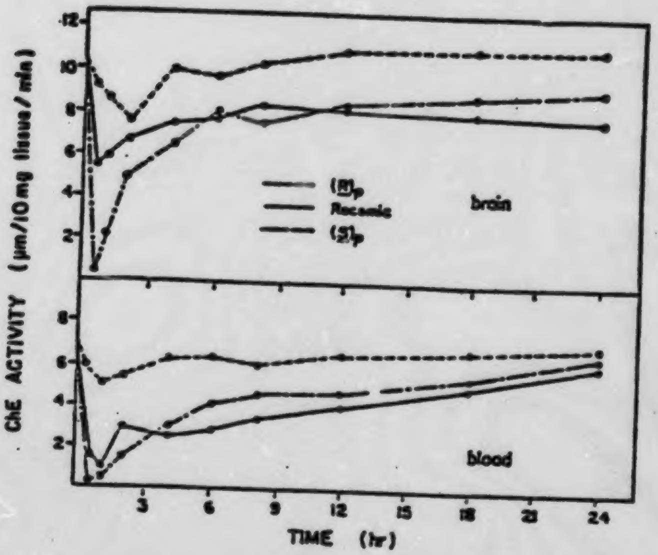


FIG. 2. Level of cholinesterase activity in mouse brain and blood at various time intervals after intraperitoneal administration of 0.6 mg/kg racemic and enantiomeric fonofos oxon. Each point represents the mean of 3 replications.

Stereoselective inhibition of brain and whole-blood ChE obtained from mice treated with 0.6 mg/kg of the enantiomers of fonofos oxon also was observed. The ChE depression curves in Fig. 2 show that the cholinesterases from mice treated with ($\underline{S}$)$_P$-oxon were inhibited at a faster rate and to a greater extent than mice treated with racemic or ($\underline{R}$)$_P$-oxon. At the same interval of 30 min after treatment, ($\underline{S}$)$_P$-oxon inhibited approximately 95% of the brain and blood ChE, while approximately 18 and 30% inhibition of the respective enzymes was observed with the enantiomeric ($\underline{R}$)$_P$-oxon. Maximum inhibition of both blood and brain ChE by ($\underline{S}$)$_P$-oxon occurred 30 min after treatment. This also appeared to be the situation with the inhibition of blood ChE by the ($\underline{R}$)$_P$-oxon, but inhibition of brain ChE was slower and 2 hr were required before maximum inhibition was attained. Beginning at the point of maximum inhibition, a gradual increase of ChE activity occurred and in general a major portion of the original cholinesterase activity was recovered after 24 hr. As in the case of fonofos, good agreement between toxicity of the enantiomers or racemate and *in vivo* ChE inhibition was evident.

Stereoselectivity in *in vivo* inhibition of HFAChE also was observed. Figure 3 presents results for the *in vivo* inhibition of HFAChE after topical application of house flies with 10 and 5 μg/g of the enantiomers and racemates of fonofos and fonofos oxon, respectively. HFAChE was inhibited at a faster rate and extent by the more toxic ($\underline{R}$)$_P$-fonofos or ($\underline{S}$)$_P$-fonofos oxon than their enantiomers. In contrast to the mouse enzymes, very small amounts of recovery or regeneration of inhibited HFAChE was observed. The results obtained from the *in vivo* ChE inhibition studies were qualitatively consistent with the results obtained from toxicity and *in vitro* ChE inhibition studies. The more toxic enantiomers of fonofos and fonofos oxon inhibited HFAChE both *in vivo* and *in vitro* to a greater extent than the less toxic enantiomer.

In Vitro Metabolism of the Chiral Isomers of Fonofos in the Presence of Mouse Liver Microsomal Mixed-Function Oxidase - Prior to the study of the chiral isomers of fonofos, the racemate was first examined to determine the conditions for maximum conversion of fonofos to fonofos oxon. Using racemic ^{14}C-phenyl fonofos, maximum enzymatic activity was found between pH 7.4 and 7.8 and 0.1 $\underline{M}$ Tris buffer was found superior to 0.1 $\underline{M}$ phosphate. A time course study showed that the amount of fonofos oxon formed increased linearly for approximately 30 min and then slowly leveled off to a plateau after 2 hr. As expected, NADPH was essential to metabolic activity. Based on the amount of fonofos being metabolized, the concentration of fonofos for maximum MFO activity was approximately 96 μ$\underline{M}$ or 120 μg per 5 ml incubation mixture. However, maximum efficiency for fonofos oxon formation was observed at a fonofos concentration of about 59 μg per 5 ml (48 μ$\underline{M}$). Therefore, this concentration was selected for subsequent experiments on the stereochemical aspects of the oxidative desulfuration reaction. Besides fonofos oxon, the other metabolic products observed from the use of ^{14}C-phenyl fonofos were diphenyl disulfide (DPDS) and minor amounts of diphenyl disulfide oxide (also see Table 5).

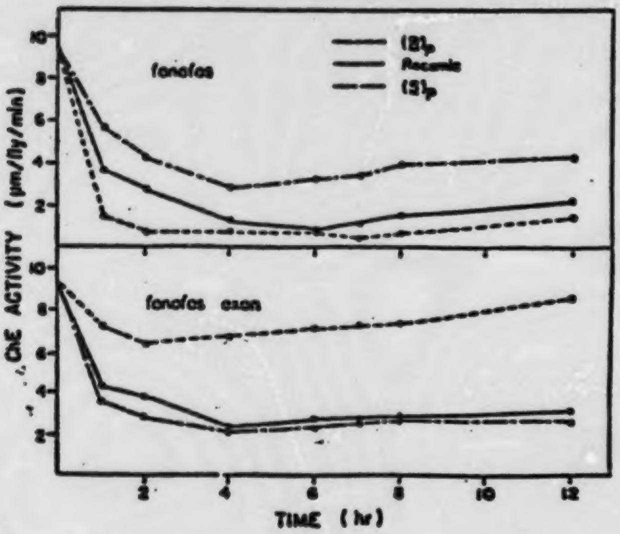


FIG. 3. Level of cholinesterase activity in house fly head at various time intervals after topical administrtion of 10 µg/g racemic and enantiomeric fonofos, and 5 µg/g racemic and enantiomeric fonofos oxon. Each point represents the mean of 3 replications.

Data for the amounts of fonofos oxon and DPDS isolated per unit weight of mouse liver microsomes (μg fonofos equivalents per mg protein) in the absence and presence of esterase inhibitors and other additives are presented in Table 5. The essentiality of NADPH in the metabolic reaction is clearly indicated and this, along with the drastic reduction in products observed in a carbon monoxide atmosphere, provided evidence that the metabolic reactions were catalyzed by an NADPH-dependent microsomal MFO enzyme system. In the control reaction, significantly larger amounts of DPDS were obtained relative to fonofos oxon. Owing to the possibility that the formation of DPDS was catalyzed by an esterase present in the microsomal fraction, i.e., via esterase-catalyzed hydrolysis of fonofos or fonofos oxon to benzenethiolate and subsequent oxidative coupling of the benzenethiolate to DPDS, the effect of a variety of esterase inhibitors on the relative yields of products was examined. Although the addition of some of these inhibitors, particularly paraoxon and bis-(p-nitrophenyl) phosphate, resulted in reversing the relative amounts of products compared to the control reaction, the magnitude of the reversal was not large enough to warrant use of inhibitors in subsequent studies with the chiral isomers. The reversal of products, however, appeared to be caused by decreased formation of DPDS, indicating that microsomal esterases contributed to a small extent to DPDS formation. Since DPDS was not formed in the absence of NADPH, esterases probably are involved in the hydrolysis of fonofos oxon but not fonofos. Comparable control experiments using ^{14}C-ethoxy-fonofos largely supported the results obtained with the ^{14}C-phenyl labeled fonofos. In this case, ethyl ethylphosphonothioic acid (ETP) and ethyl ethylphosphonic acid (EOP), besides fonofos oxon, were obtained as metabolic products. In the absence of NADPH, no ETP or EOP was detected, again providing support for an oxidative pathway for the formation of degradation products as well as oxon.

After establishment of optimum conditions for conversion of fonofos to fonofos oxon, the stereochemistry of the oxidative desulfuration of (S)p-and (R)p-fonofos was investigated. Two separate sets of 300 individual incubations (59 μg enantiomers per incubation) with (S)p-fonofos and a single set of the same number of incubations with (R)p-fonofos were carried out. From a total of 17.5 mg (S)p-fonofos (300 X 59 μg) approximately 3 mg of fonofos oxon was isolated from each set. A larger yield of 5.3 mg oxon was obtained from the (R)p-fonofos. The oxons obtained from each fonofos enantiomer were determined to be >99% pure (disregarding isomeric content) as estimated by autoradiography of a TLC plate and by GLC analysis. Optical rotation measurements in cyclohexane solvent using a 1 dm cell gave the following values for each oxon: from (S)p-fonofos, +0.148° (2.98 mg) and +0.190° (3.07 mg); from (R)p-fonofos, -0.369° (5.33 mg). Calculated specific rotations ($[\alpha]_D^{25}$) are presented in Table 6, along with those of the starting fonofos enantiomers. The degree of stereospecificity of the desulfuration reaction was calculated by comparing the specific rotations of the isolated oxons with those of the oxons [(R)p +112.64° (c 1.02), (S)p -121.70° (c 0.636)] which were

TABLE 5. EFFECT OF COFACTORS AND INHIBITORS ON THE IN VITRO MICROSOMAL OXIDATION OF RACEMIC ^{14}C-PHENYL FONOFOS.

	Amounts of metabolic products (μg fonofos equivalent/mg protein)			
	Oxon	DPDS	DPDS-oxide	Total
Control[a]	0.76[b]	0.85	0.07	1.68
-NADPH	-	-	-	-
CO-Air (1:1)	0.07	0.02	0.02	0.11
+ p-Nitrophenyl phosphate (1 mM)	0.66	0.62	0.02	1.30
+ Bis-p-nitrophenyl phosphate (1 mM)	0.80	0.65	0.02	1.47
+ Tri-p-nitrophenyl phosphate (1 mM)	0.71	0.85	0.04	1.60
+ Paraoxon	0.81	0.64	0.03	1.48
+ Tributyl phosphorotrithioate (1 mM)	0.44	0.40	0.04	0.88

Note a. The standard incubation mixture contained 0.1 M Tris buffer pH 7.8, G-6-P (1 mM), NADPH (0.5 mM), G-6-P dH (1 EU), mouse liver microsomal suspension (17.5 mg) and fonofos enantiomer (59 μg). Incubation was carried out at 37.5° for 2 hr.

Note b. Each value represents the average of two replications.

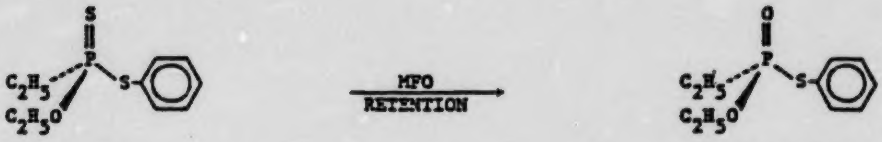
TABLE 6. SPECIFIC ROTATION OF STARTING $(R)_P$- AND $(S)_P$-FONOFOS AND RESPECTIVE OXONS ISOLATED AFTER INCUBATION OF EACH FONOFOS ISOMER WITH MOUSE LIVER MICROSOMAL MIXED-FUNCTION OXIDASE.

Fonofos	$[\alpha]_D^{25}$[a]	Fonofos oxon	$[\alpha]_D^{25}$	Stereospecificity
$(S)_P$	+ 138.40° (c 0.301)	$(R)_P$	+ 49.78° (c 0.298)	72% retention
$(S)_P$	+ 138.40°	$(R)_P$	+ 61.83° (c 0.307)	72% retention
$(R)_P$	- 114.56° (c 0.309)	$(S)_P$	- 69.29° (c 0.533)	79% retention

Note a. Rotations were determined in a Rudolph high precision polarimeter at 25° using a 1 dm cell, cyclohexane solvent, and sodium D line.

synthesized from the respective enantiomers of O-ethyl ethylphosphonothioic acid. Owing to differences in the specific rotations of the synthesized isomers of fonofos and fonofos oxon, these values must be regarded as estimates.

The results provided conclusive evidence that mouse liver MFO catalyzed conversion of fonofos to fonofos oxon proceeded predominantly with retention of configuration, as depicted below with (S)p-fonofos.



(S)$_p$-fonofos

(R)$_p$-fonofos oxon

Stereoselective Rate Differences in Metabolism - Because of the general phenomenon of stereoselectivity in enzyme interactions with chiral substrates and inhibitors, the effect of chirality on the relative susceptibilities of the fonofos enantiomers to oxidative metabolism was investigated. The relative amounts of fonofos isomers metabolized under optimal and identical conditions of mouse liver MFO activity after different time intervals are presented graphically in Fig. 4. The plots clearly reveal a faster rate of metabolism for (R)p- than for (S)p-fonofos.

Similar plots showing the relation between time and amounts of DPDS and oxon obtained from each fonofos enantiomer and racemate are presented in Figs. 5 and 6, respectively. Consistent with the values for the disappearance of fonofos, larger amounts of both DPDS and fonofos oxon were obtained at each interval from (R)p- than from (S)p-fonofos. Based on the plots in Figs. 5 and 6, it appears that the substantial difference in the rate of metabolism of the fonofos isomers is attributable more to the difference in the reaction leading to DPDS than to fonofos oxon. Thus, while (R)p-fonofos was metabolized at a faster rate than its enantiomer, a significantly larger fraction of the product was in the form of degraded material.

Further evidence confirming the greater susceptibility to metabolism of (R)p-fonofos compared to its enantiomer was found in studies with racemic ^{14}C-fonofos. When the racemate was incubated with liver MFO, the unmetabolized fonofos which was recovered gave a specific rotation of $[\alpha]_D^{25}$ +7.63° (c 1.704 in cyclohexane). By assuming a specific rotation of +138.4° for (S)p-fonofos, the calculated ratio of (S)p- to (R)p-fonofos in the recovered material was approximately 53/47.

Effect of Esterases on Fonofos Chiral Isomers - In seeking a satisfactory explanation for the toxicity differences between the chiral isomers of fonofos and fonofos oxon, the possible impact of differences in hydrolytic degradation also was considered. Data for the relative amounts of DPDS produced after incubation for 1 hr of the various ^{35}S-enantiomers with

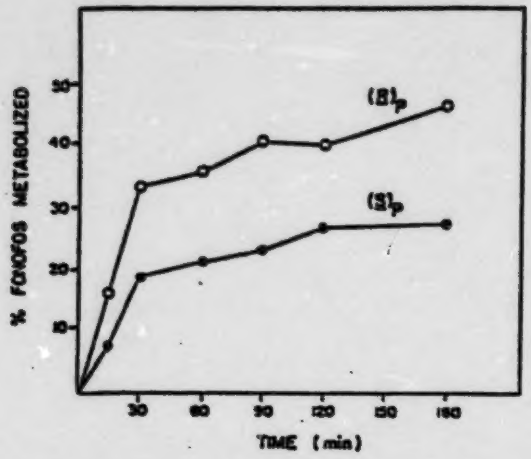


Fig. 4. Time course of the *in vitro* metabolism of (*S*)$_P$- and (*R*)$_P$-fonofos by mouse liver microsomal mixed-function oxidase system. Each value represents the average of two replications.

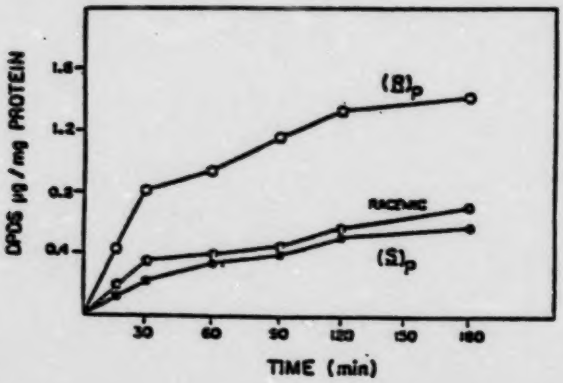


Fig. 5. Time course of the formation of diphenyl disulfide (DPDS) in µg fonofos equivalent for the *in vitro* metabolism of racemic (*S*)$_P$- and (*R*)$_P$-fonofos by mouse liver microsomal mixed-function oxidase. Each value represents the average of two replications.

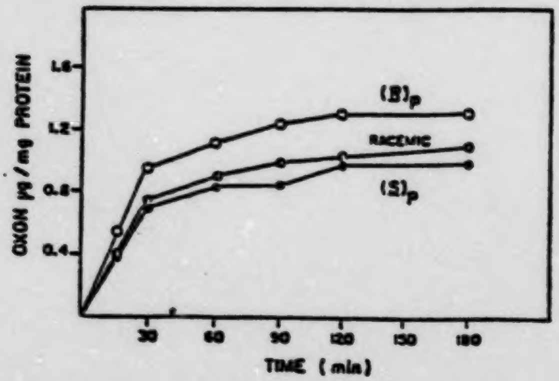


Fig. 6. Time course of the formation of fonofos oxon in µg fonofos equivalent for the *in vitro* metabolism of racemic (◐), (*S*)$_P$- (●), and (*R*)$_P$-fonofos (○) by mouse liver microsomal mixed-function oxidase. Each value represents the average of two replications.

mouse liver homogenate and microsome, rat and mouse blood serum are presented in Table 7. Enzyme incubations were carried out in 0.1 M Tris buffer at pH 8.0. In the absence of NADPH, neither mouse liver homogenate nor microsome had any effect on fonofos degradation, in agreement with preceding studies on the oxidative activation and degradation of fonofos. Further, fonofos also was stable to the action of mouse and rat serum both in the presence and absence of possible cofactors.

In contrast to fonofos, appreciable degradation of fonofos oxon to DPDS took place in the presence of mouse and rat serum. Significant amounts of DPDS also were formed after incubation of the oxon with liver homogenate and microsomal fraction, particularly when NADPH was present. In all cases, ^{35}S-DPDS was the major product observed although two other metabolites of minor significance occasionally were detected. The identity of these metabolites is not known. Although degradation of fonofos oxon to DPDS by mouse and rat serum was slow compared to the oxidative reactions mediated by MFO, the results revealed that (S)p-fonofos oxon was hydrolyzed at a faster rate than its (R)p enantiomer. Plots showing differences in the rate of formation of DPDS obtained from each isomer after incubation with rat serum are given in Fig. 7. From the slopes (S)p-fonofos oxon evidently was hydrolyzed at a rate more than 2-fold faster than its enantiomer. At the 60-min incubation period, 2.76% of the applied radioactivity was converted to DPDS from (S)p-fonofos oxon, compared to 1.19% from the (R)p enantiomer.

Metabolism in the House Fly - House flies were treated at 6.5 μg/g of each of the fonofos enantiomers, a dosage which is approximately the LD_{50} for the more toxic (R)p isomer and sublethal for the (S)p isomer. At this dosage, about 20% of the house flies treated with (R)p-fonofos were down 6 hr after treatment and the remaining flies showed symptoms of cholinergic poisoning. No intoxication symptoms were obvious in flies treated with the (S)p isomer.

The rate of fonofos penetration was estimated from the amount of radioactivity recovered from the external surface of treated flies and also from the amounts present internally in the fly. The fonofos enantiomers were rapidly absorbed by the flies but no significant difference in penetration rates between the two enantiomers was observed.

House flies were examined for fonofos metabolism 4 hr after topical treatment with 6.5 μg/g ^{35}S-phenyl labeled enantiomer. The external wash, organo-soluble and water-soluble fractions of the fly homogenate were analyzed separately. Data summarizing the nature and amounts of metabolites obtained from the flies are presented in Table 8. Unchanged fonofos was the principal constituent in the external wash, along with lesser amounts of fonofos oxon, methyl phenyl sulfoxide and sulfone, and a substantial amount of polar material which remained at the origin of the TLC plate. The polar material was not identified but it probably was a conjugated metabolite coming from the fly excreta. Fonofos also was the major constituent present internally in the flies, most of it being

TABLE 7. EFFECT OF ENZYME AND COFACTOR ON THE DEGRADATION OF PHENYL-^{35}S-FONOFOS AND FONOFOS OXON TO DIPHENYL DISULFIDE.

	Diphenyl disulfide in % of applied radioactivity[a]			
	$(\underline{S})_p$-Fonofos	$(\underline{R})_p$-Fonofos	$(\underline{S})_p$-Oxon	$(\underline{R})_p$-Oxon
Mouse liver homogenate[b]				
+ NADPH[c]	12.42	20.65	0.58	0.39
- NADPH	N.D.[d]	N.D.	0.15	0.13
Mouse liver microsome				
+ NADPH	14.87	26.74	0.49	0.44
- NADPH	N.D.	N.D.	<0.1	<0.1
Mouse serum	N.D.	N.D.	0.95	0.58
Rat serum	N.D.	N.D.	2.76	1.19

Note a. The data are the average of two separate experiments; each experiment consisted of two replications.

Note b. Amounts of liver and serum protein per incubation are as follows: mouse liver homogenate - 25 mg; mouse liver microsome - 17 mg; rat and mouse serum - 8 mg.

Note c. Final concentration of NADPH was 1 m*M*.

Note d. Not detected.

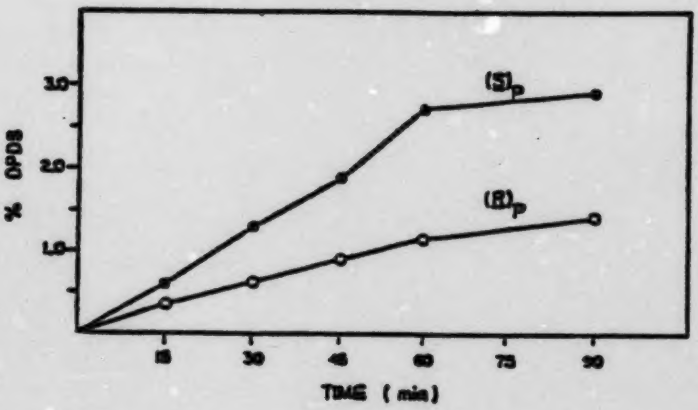


FIG. 7. Time course of the formation of diphenyl disulfide (DPDS) in percent of applied radioactivity for the *in vitro* metabolism of $(\underline{S})$p- and $(\underline{R})$p-fonofos oxon by rat serum esterase. Each value represents the average of two replications.

TABLE 8. NATURE AND AMOUNTS OF METABOLITES IN VARIOUS FRACTIONS FROM HOUSE FLIES TREATED WITH 6.5 μg/g PHENYL-^{35}S-FONOFOS ENANTIOMERS.

	% of Applied Radioactivity	
	$(\underline{S})_P$-fonofos	$(\underline{R})_P$-fonofos
External wash		
Fonofos	19.3	21.6
Fonofos oxon	0.7	0.6
Methyl phenyl sulfone	0.5	0.2
Methyl phenyl sulfoxide	5.0	1.7
Origin	9.6	10.9
TOTAL	35.1	35.0
Fly homogenate - Organo-soluble		
Fonofos	28.5	36.1
Methyl phenyl sulfone	1.9	1.4
Origin	1.8	2.1
TOTAL	32.2	39.6
- Water-soluble		
Diphenyl disulfide	0.4	1.2
Fonofos	0.4	3.1
Methyl phenyl sulfone	1.3	1.0
Methyl phenyl sulfoxide	1.7	1.3
3-OH-Phenyl methyl sulfone	0.8	0.7
4-OH-Phenyl methyl sulfone	12.8	6.5
Origin	4.0	1.6
TOTAL	21.4	15.4
Unextractable Radioactivity	7.6	6.4
TOTAL RECOVERY	96.3	96.4

recovered in the organo-soluble fraction along with methyl phenyl sulfone. Water-soluble metabolites were isolated and identified after subjecting the aqueous phase to acid hydrolysis. In addition to the same metabolites found in the other fractions, 3-hydroxyphenyl and 4-hydroxyphenyl methyl sulfones also were detected, the latter being present in greatest abundance. Total recovery of radioactivity was about 96% with both enantiomers.

On the whole, the metabolism of the fonofos enantiomers was qualitatively and quantitatively similar. However, based on the relative amounts of fonofos and 4-hydroxyphenyl methyl sulfone isolated, the less toxic (S)p-fonofos appeared to be metabolized to a greater extent during the 4-hr holding period than the (R)p isomer. The difference is probably attributable to the physical state of the flies, i.e., those treated with (R)p-fonofos were visibly intoxicated while those treated with the (S)p isomer appeared to be normal.

Metabolism of Fonofos in the White Mouse - Mice were treated orally at two dosages, 4 and 8 mg/kg. At 8 mg/kg (R)p-fonofos, mice showed severe poisoning symptoms 4 hr after treatment and remained in an intoxicated state during the entire 96 hr metabolism holding period. A total of 5 mice were treated with (R)p-fonofos and of these only 2 survived the 96 hr holding period. Mice treated with 8 mg/kg (S)p-fonofos and 4 mg/kg (R)p-fonofos showed mild symptoms of cholinergic poisoning but recovered after 12 hr. Mice treated with 4 mg/kg (S)p-fonofos were essentially unaffected. The oral LD_{50} values for (R)p- and (S)p-fonofos are 9.5 and 32 mg/kg, respectively.

The rate of excretion of administered radioactivity was monitored in both urine and feces. The results are presented in Fig. 8 for the 2 dosages of 8 and 4 mg/kg. The figures show that at both dosages radioactivity associated with the less toxic (S)p isomer was eliminated faster from the mouse than its enantiomer. At the higher dosage of 8 mg/kg, about 95% of administered (S)p isomer was eliminated within the 96-hr holding period compared to about 50% for the more toxic (R)p isomer. The major route of elimination was *via* the urine. At the lower dosage of 4 mg/kg, the initial rate of elimination was substantially smaller for the (R)p isomer, but the rate gradually increased during the holding period and the total recovery of administered radioactivity for the two isomers was about the same after 96 hr. About 3-fold more radioactivity was eliminated from the mouse treated with the (S)p isomer than its enantiomer during the first 24 hr after treatment.

Table 9 contains data on the nature and total amounts of ^{35}S-labeled metabolites present in the urine and feces of mice 96 hr after treatment with the fonofos isomers at the two dosages. The expected metabolites were found, including unchanged fonofos, fonofos oxon, methyl phenyl sulfoxide in the organo-soluble fraction, and 3-hydroxyphenyl and 4-hydroxyphenyl methyl sulfone in the aqueous fraction. Small amounts of diphenyl disulfide and diphenyl disulfide oxide also were detected in the organo-soluble phase. These metabolites were not reported in previous

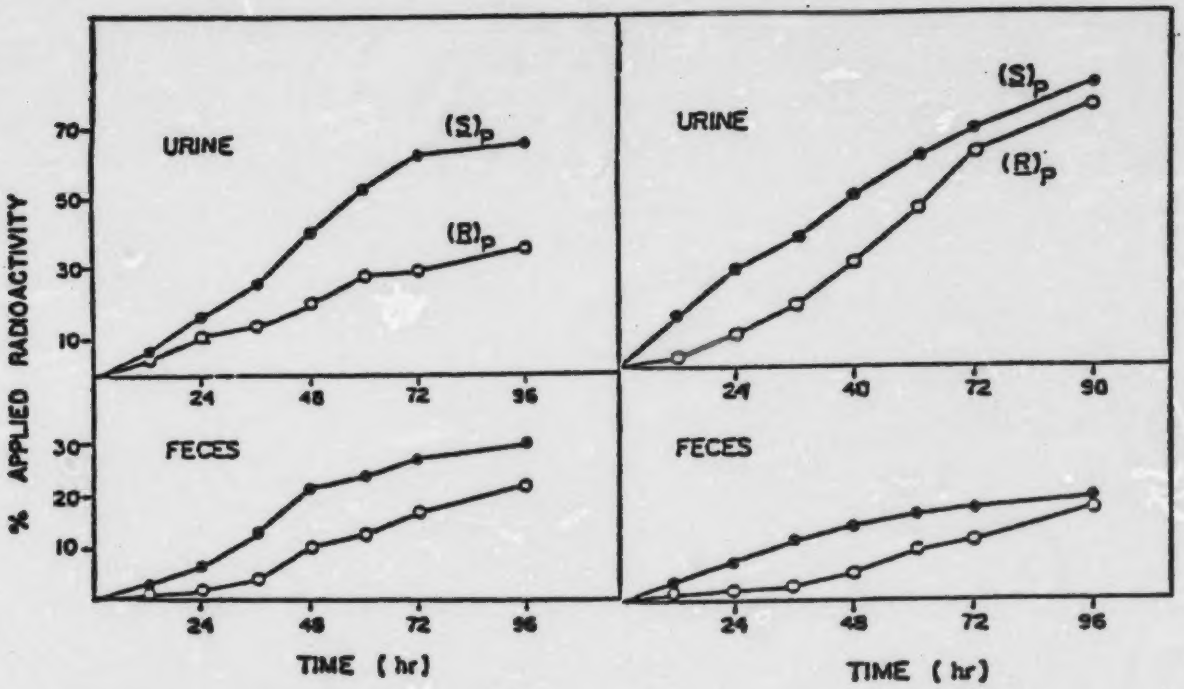


FIG. 8. Plots showing the percentage of applied radioactivity present in urine and feces obtained from female white mice at different time intervals following oral administration of 8 mg/kg (left) and 4 mg/kg (right) (S)p-fonofos and (R)p-fonofos.

TABLE 9. TOTAL FONOFOS AND METABOLITES ISOLATED FROM THE URINE AND FECES OF MICE 96 HR AFTER TREATMENT WITH 4 mg/kg AND 8 mg/kg ENANTIOMERS OF PHENYL-^{35}S-FONOFOS.

	% of Applied Radioactivity			
	4 mg/kg		8 mg/kg	
	$(\underline{S})_P$	$(\underline{R})_P$	$(\underline{S})_P$	$(\underline{R})_P$
Diphenyl disulfide	-	-	0.28	-
Fonofos	0.53	0.76	2.67	3.06
Fonofos oxon	0.04	0.65	0.11	0.18
Diphenyl disulfide oxide	0.50	0.75	0.22	-
Methyl phenyl sulfoxide	4.65	3.89	11.08	4.14
Methyl phenyl sulfone	17.72	18.25	16.43	8.58
4-OH-Phenyl methyl sulfone	15.30	14.15	13.85	8.70
3-OH-Phenyl methyl sulfone	13.16	13.40	15.69	7.74
Origin	26.30	28.75	17.60	12.20
Unextractable (urine)	16.10	14.70	14.67	8.42
TOTAL	94.3	95.3	92.6	53.0

studies with rats and racemic fonofos.[2,3] A relatively large amount of radioactivity remained as a polar unknown at the origin of the TLC plate or as unextractable water-soluble materials even after acid hydrolysis. These probably were degraded and conjugated products of fonofos.

The water-soluble fraction contained large amounts of 3- and 4-hydroxyphenyl methyl sulfone. These were excreted as conjugated products and were liberated by acid hydrolysis. These metabolites were reported previously by McBain et al.[2,4]

At the lower dosage of 4 mg/kg, very little difference in the metabolism of (R)p- and (S)p-fonofos was observed. For all practical purposes, the amounts of each metabolite obtained from the fonofos isomers after treatment at 4 mg/kg are the same. However, notably lesser amounts of metabolites were formed from (R)p-fonofos than from its enantiomer at 8 mg/kg. This is doubtlessly attributable to the severely intoxicated state of the mouse treated with the (R)p isomer. Evidently, the normal excretory and metabolic functions of the animal were inhibited.

Metabolism of Fonofos Oxon in Mice - Mice were treated orally with 4 mg/kg ^{35}S-phenyl labeled (S)p- and (R)p-fonofos oxon. This dosage is sublethal for the (R)p-oxon and is approximately the LD_{20} value for the more toxic (S)p isomer. The LD_{50} values of (S)p- and (R)p-fonofos oxon are 6 and 38 mg/kg, respectively. Animals treated with the (S)p isomer showed cholinergic poisoning symptoms but recovered after 12 hr. Animals treated with (R)p-oxon were normal.

As in the case of fonofos, differences in the excretion rates of radioactivity from mice treated with the two oxon isomers also were observed (Fig. 9). The major route of excretion was via the urine. Excretion was slower in the mouse treated with the more toxic (S)p isomer and the total recovery of administered radioactivity at the end of 96 hr was 56%. In comparison, 89% of the administered (R)p isomer was recovered. Again, the difference in elimination rates is probably a result of the different toxicological conditions of the mice.

Table 10 presents data on the nature and amounts of ^{35}S-labeled metabolites in the urine and feces of mice treated with the oxon enantiomers. The metabolites identified were methyl phenyl sulfoxide and sulfone, and 3- or 4-hydroxyphenyl methyl sulfone, along with a small amount of unchanged oxon. Diphenyl disulfide and diphenyl disulfide oxide which were found in the fonofos metabolism studies were not detected. Because of the larger amount of radioactivity excreted from mice treated with (R)p-oxon, proportionately larger amounts of each metabolite were recovered from (R)p- than from (S)p-oxon treated mice. The relative amounts of metabolites, i.e., relative to the total amount of radioactivity excreted, remained fairly constant and there appeared to be little difference in the pattern of metabolism between the two enantiomers.

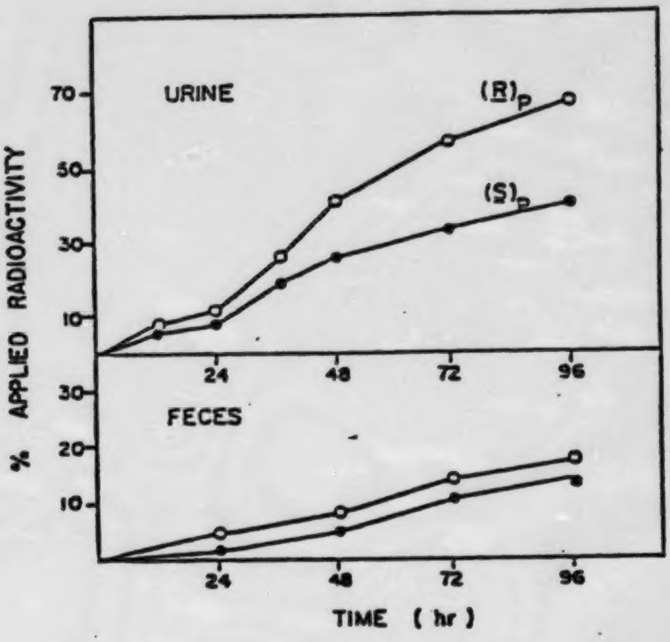


FIG. 9. Plots showing the percentage of applied radioactivity present in urine and feces obtained from female white mice at different time intervals following oral administration of 4 mg/kg ^{35}S-(S)p (●) and (R)p (o) isomers of fonofos oxon.

TABLE 10. NATURE AND AMOUNTS OF METABOLITES RECOVERED FROM URINE AND FECES OF WHITE MICE TREATED WITH 4 mg/kg OF EACH ENANTIOMER OF PHENYL-^{35}S-FONOFOS OXON.

	% of Applied Radioactivity			
	Urine		Feces	
	$(\underline{S})_P$	$(\underline{R})_P$	$(\underline{S})_P$	$(\underline{R})_P$
Organo-soluble				
Fonofos oxon	0.54	0.38	0.14	-
Methyl phenyl sulfoxide	1.75	3.62	0.60	0.09
Methyl phenyl sulfone	3.90	9.34	0.86	2.16
Origin	0.24	2.48	2.05	1.93
TOTAL	6.43	15.82	3.65	4.18
Water-soluble				
4-OH-Phenyl methyl sulfone	11.15	19.75	2.84	3.76
3-OH-Phenyl methyl sulfone	9.49	14.91	2.43	3.14
Origin	6.46	8.56	3.58	4.30
TOTAL	27.10	43.22	8.85	11.20
Total Unextractable	6.82	9.72	3.75	4.77
Total Recovery	40.35	68.76	16.25	20.15

Total recovery of applied radioactivity

$(\underline{S})_P$-fonofos oxon 56.6%

$(\underline{R})_P$-fonofos oxon 88.9%

Absorption and Metabolism of the Fonofos Isomers in the Corn and Cotton Plants - Phenyl-^{35}S-fonofos was rapidly absorbed by corn and cotton plants from the nutrient solution by root uptake. The disappearance of each fonofos enantiomer from the nutrient solution was monitored at 12- and 24-hr intervals for 4 days. The results are summarized in Fig. 10 which shows the relative amounts of each isomer absorbed by the cotton or corn plant with increasing time intervals. In both plant species, (S)p-fonofos was absorbed at a faster initial rate and to an overall greater extent than the (R)p enantiomer. For example, 12 hr following exposure of corn roots to the nutrient solution containing radiolabeled fonofos, about half as much (R)p enantiomer was taken up by the plant compared to the (S)p enantiomer. A similar order of stereoselectivity was observed for the cotton plant. Even after 4 days following exposure more (R)p-fonofos was recovered from the nutrient solution than (S)p-fonofos, but the difference was much smaller than at 12 hr. The loss of radioactivity in a control medium, i.e., nutrient solution without plants, was negligible and, also, fonofos was stable throughout the test period in the nutrient solution.

The stereoselective absorption of (S)p-fonofos over the (R)p enantiomer by plants was also demonstrated in a separate experiment using racemic phenyl-^{14}C-fonofos. Corn plants were allowed to stand in hydroponic media containing 3.2 ppm racemic phenyl-^{14}C-fonofos for 7 days. The unabsorbed fonofos remaining in solution was extracted and purified, and was shown to have optical activity, $[\alpha]_D^{25}$ -19.25 (c 0.265 in cyclohexane). The negative sign of rotation is indicative of a larger (R)p- to (S)p-fonofos ratio remaining in the hydroponic phase, a finding which clearly demonstrates preferential absorption of the (S)p isomer.

The nature and amounts of the various metabolites isolated from corn and cotton plants 5 days following continuous exposure to the fonofos enantiomers by root treatment are indicated in Table 11. Duplicate experiments were carried out and the results in this table represent the average of two separate studies. Among the different compounds isolated from the plants, unchanged fonofos was by far of greatest significance, ranging from 32.6% to 63.3% of the applied radioactivity. Of the two isomers, (S)p-fonofos was recovered in greater quantities from both plants, indicating greater susceptibility of (R)p-fonofos to metabolic alteration. This is evident also from the larger quantities of degradation products, e.g., DPDS-oxide, MPSO and $MPSO_2$, recovered from plants treated with the (R)p isomer.

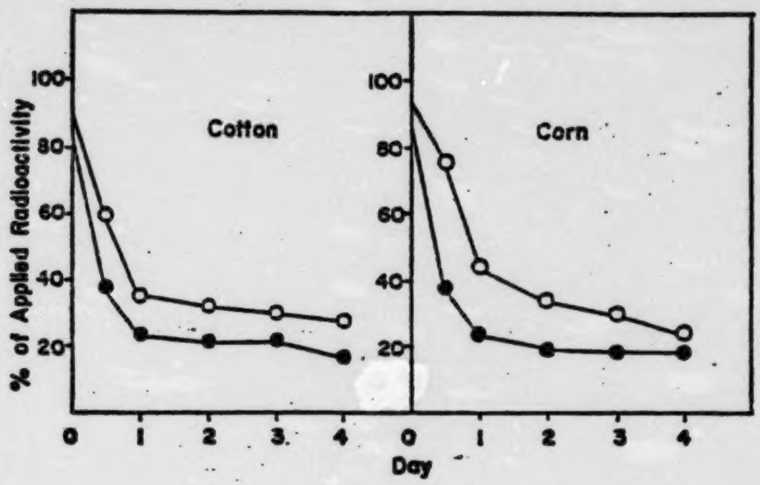


FIG. 10. Distribution of applied radioactivity remaining in the nutrient solution at different time intervals following treatment with (S)p-phenyl-^{35}S-fonofos (●——●) and (R)p-phenyl-^{35}S-fonofos (o——o) in the root uptake study.

TABLE 11. NATURE AND AMOUNT OF CHIRAL PHENYL-^{35}S-FONOFOS ENANTIOMERS IN THE ORGANIC SOLVENT EXTRACTABLE, WATER SOLUBLE AND BOUND FRACTIONS IN CORN AND COTTON PLANTS FIVE DAYS AFTER ROOT APPLICATION.

	Percent Applied Radioactivity			
	Cotton		Corn	
Organic Solvent Extractable	(*S*)p-fonofos	(*R*)p-fonofos	(*S*)p-fonofos	(*R*)p-fonofos
Fonofos	48.3	32.6	63.3	41.9
Fonofos oxon	0.8	0.4	N.D.*	N.D.
DPDS-oxide	2.2	6.8	4.1	12.0
MPSO	1.6	5.8	0.9	3.0
$MPSO_2$	8.0	13.7	1.8	10.5
Origin	0.2	0.3	1.7	1.1
Total	61.1	59.6	71.8	68.5
Water soluble	11.8	6.5	10.2	9.6
Bound residue	7.1	9.4	1.0	6.1
Total recovery	80.0	75.5	83.0	84.2

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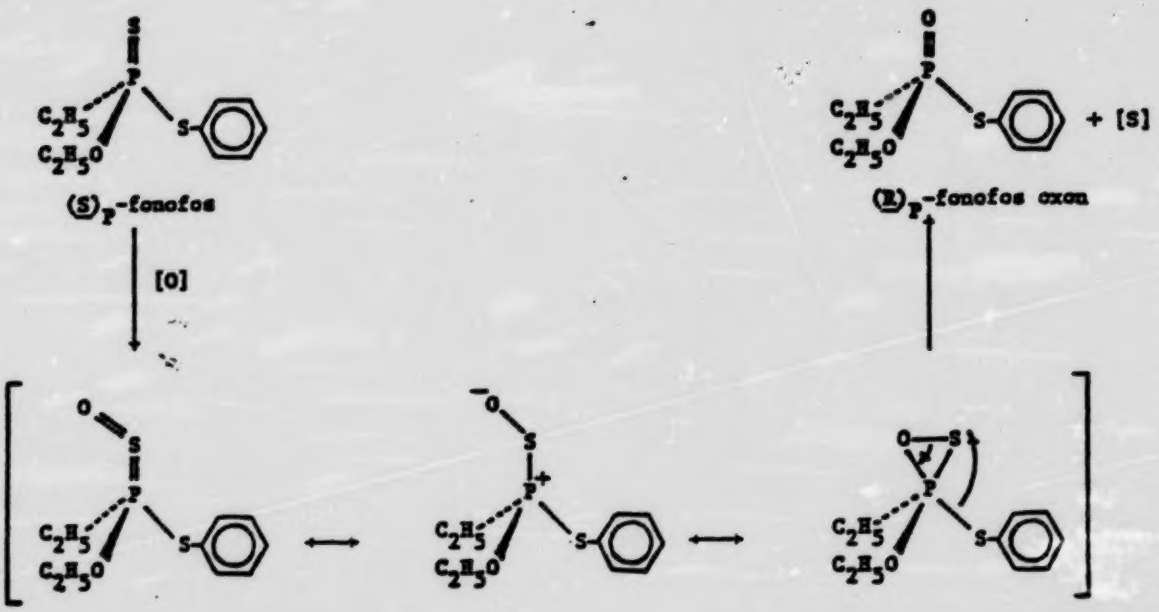
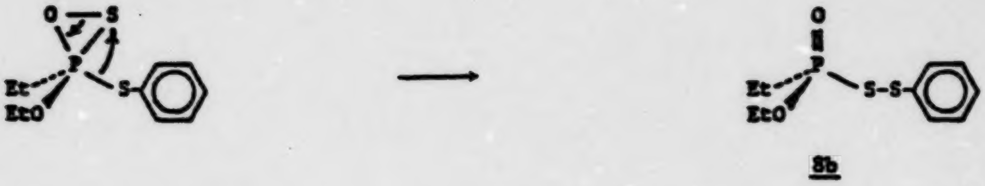
*N.D. = not detectable

Summary - The elucidation of the stereochemical course of the MFO-catalyzed conversion of fonofos to fonofos oxon was essential to the explanation of the order of toxicity of the fonofos isomers and was also of theoretical significance. Based on the stereochemical results, a mechanism consistent with the preferential retention of configuration observed for the desulfuration reaction may be suggested. The mechanism is similar to that proposed by Ptashne and Neal[5] for the MFO-catalyzed conversion of parathion to paraoxon in which initial attack by oxygen on the thiono-sulfur to give the S-oxide was postulated. Closure of the S-oxide to the three-membered ring with subsequent loss of sulfur gives the oxon with retention of configuration.



The conversion of (S)p-fonofos (4a) to (R)p-fonofos oxon (6b) by the action to m-chloroperoxybenzoic acid occurred predominantly with retention of configuration of the phosphorus atom. In contrast, oxidation to the rearrangement product, (R)p-phenyl ethyl(ethoxy) phosphinyl disulfide (8b) took place with predominant inversion of configuration. In the case of the reaction leading to the oxon, the stereochemical course was identical to that observed with mouse liver MFO. While it is possible that fonofos oxon and phenyl ethyl(ethoxy)phosphinyl disulfide are formed by two separate reactions between fonofos and m-chloroperoxybenzoic acid, the same 3-membered ring intermediate also may account for the stereochemical result as indicated below.



Thus, the stereochemistry of both oxidation products may readily be accounted for by the three-membered ring intermediate. This intermediate is possible by considering hybridization of an sp^3 orbital with a d_{xy} orbital, giving two spd bonds at 71° and three sp^3 bonds[6]. Hybridization of this type is compatible with the angular strain associated with a three-membered ring.

The toxicological data in Table 3, indicating 2- to 4-fold higher toxicity of (R)p-fonofos to mice and house flies relative to (S)p-fonofos, was of considerable significance since it implied that the oxon was generated in the animal with some degree of stereospecificity. Compared to fonofos, the difference in toxicity between the fonofos oxon enantiomers was greater and (S)p-oxon was 2.6- (i.p.) and 12-fold more toxic to white mice and house flies, respectively, than the (R)p-oxon.

The relative toxicities of (S)p- and (R)p-fonofos oxon to house flies and white mice were qualitatively in agreement with their relative in vitro anticholinesterase activities toward fly-head, mouse brain, and blood ChE, i.e., the isomer of higher toxicity was more effective in inhibiting the three enzymes. On a quantitative basis, however, the differences in anticholinesterase activities between the isomers were substantially greater (49- to 60-fold) than the differences in toxicities to house flies and white mice. Since the two isomers differ only in chirality at the phosphorus atom, perhaps a closer relationship between toxicity and anticholinesterase activity might be expected. Although it is difficult to arrive at a quantitative relationship, it appears that in vivo ChE inhibition is more closely correlated with toxicity (see Figs. 1 and 2). At the same intraperitoneal dosage of oxon isomers (0.6 mg/kg), the level of maximum inhibition of both brain and blood ChE obtained from mice treated with (S)p oxon was approximately 3-fold greater than enzymes obtained from mice treated with the (R)p enantiomer. Maximum inhibition, however, occurred at different time intervals after treatment and the relationship was further complicated by the differences in recovery rates of the inhibited enzymes. Nevertheless, agreement between in vivo inhibition of mouse ChE and intraperitoneal toxicity was good.

The relationship between in vivo inhibition of HFAChE and toxicity of house flies treated with the two isomers was less satisfactory. Maximum reduction of HFAChE in flies treated with 5 µg/g (R)p-oxon was about 35% compared to about 80% for flies treated with the same amount of (*S*)p-oxon, a slightly greater than 2-fold difference (see Fig. 3). This difference is substantially smaller than the 12-fold difference observed for house fly toxicity. Examination of the plots in Fig. 3 shows that HFAChE inhibited by (*R*)p-oxon recovered to a significantly greater extent (compare activities at 12 hr) and it is possible that ChE depression over longer time periods should be considered in relating *in vivo* inhibition with toxicity.

The smaller differences in fly and mouse toxicities between (*R*)p- and (*S*)p-fonofos may be justified with less difficulty. It is well known that thionate esters in the pure state are poor anticholinesterases and metabolic activation to the corresponding P=O ester is required for intoxication. Studies on the *in vitro* metabolism of the two isomers in the presence of mouse liver microsomal mixed-function oxidase showed that (*R*)p-fonofos and (*S*)p-fonofos were converted to the respective (*S*)p-oxon and (*R*)p-oxon with 70-80% stereospecificity. Since (*S*)p-oxon is a stronger anticholinesterase and is more toxic than (*R*)p-oxon, the order of toxicity of the fonofos isomers is in the right direction. Although metabolic conversion of fonofos to the oxon occurred with predominantly retention of configuration, apparently a significant amount of inversion also took place, leading to partial racemization of the oxon. This undoubtedly contributed to the smaller toxicity ratios between the fonofos isomers.

Further insight into the explanation for the relative toxicities of the fonofos and fonofos oxon isomers was obtained from studies on the relative rates of metabolism. While the results revealed that (*R*)p-fonofos was metabolized at a rate approximately 2-fold greater than (*S*)p-fonofos, analysis of the products showed that (*R*)p-fonofos was converted in greater proportion to the detoxication product DPDS than its enantiomer. Thus, even though (*R*)p-fonofos was metabolized by mouse liver MFO at a faster rate, the difference in amounts of fonofos oxon formed from the fonofos isomers was not large.

Rat and mouse serum were effective in degrading fonofos oxon but not fonofos. Hydrolysis of the enzyme probably was effected by an esterase, e.g., the paraoxon hydrolyzing A-esterase whose presence has been demonstrated in mammalian serum.[7,8] The *in vitro* degradation of fonofos oxon by rat or mouse serum was relatively slow compared to the oxidative reactions, but the results clearly showed that (*S*)p-oxon was degraded at a faster rate than the (*R*)p-oxon (approximately 2-fold). Although *in vitro* results do not necessarily represent events which take place *in vivo*, the greater susceptibility of (*S*)p-oxon to enzymatic degradation may account for the smaller difference in toxicities between the fonofos-oxon enantiomers relative to their differences in anticholinesterase activities (50- to 60-fold). Further, since (*R*)p-fonofos is preferentially metabolized to (*S*)p-oxon and (*S*)p-fonofos to (*R*)p-oxon, the faster rate of degradation of (*S*)p-oxon may contribute to the even smaller differences in toxicities of the fonofos enantiomers. Thus, the smaller difference in toxicities between the fonofos enantiomers compared to the relative toxicities of the oxons and their anticholinesterase activities may be accounted for by partial racemization in the oxidative desulfuration reaction and faster rate of degradation of (S)p-oxon.

2. Studies on the Mode of Action of Methamidophos.

Introduction - O,S-Dimethyl phosphoramidothioate (methamidophos) is an outstanding insecticide which is currently being marketed as a broad spectrum insecticide. Although it is a highly effective insecticide, with a bimolecular inhibition constant of (ki) $9.2 \times 10^{2} M^{-1} min^{-1}$ for the inhibition of fly head AChE, it is a relatively weak anticholinesterase.

In previous work from this laboratory[9], we found a direct relationship between toxicity to the house fly and reactivity of the phosphoramidothiolates as expressed by hydrolysis rates and an overall trend indicating direct correlation between toxicity and anticholinesterase activity. In spite of these correlations, we were unable to rationalize the comparatively high toxicities of these compounds in light of their moderate anticholinesterase activities, particularly since none of the most active compounds contained functional groups which would likely be metabolized *in vivo* to produce more active inhibitors. Therefore, additional studies on the toxicological properties, *in vitro* and *in vivo* anticholinesterase behavior, and metabolism of methamidophos were conducted.

Cholinesterase Inhibition - The results of ChE-inhibition determination with methamidophos are shown in Table 12. Methamidophos is only a moderate ChE inhibitor in comparison to other organophosphates of comparable toxicity. Further, it shows essentially no selectivity among the three sources of enzyme. This is perhaps to be expected since it contains no moiety which might be expected to confer selectivity.

TABLE 12. ANTICHOLINESTERASE ACTIVITY OF METHAMIDOPHOS.

Cholinesterase Source	I_{50} (M)
House fly head	1.1×10^{-5}
Honey bee worker head	3.5×10^{-5}
White mouse brain	8.2×10^{-6}

Aliesterase Inhibition - Many organophosphorus compounds are potent inhibitors of aliesterases as well as cholinesterase.[10] Although their inhibition of AliE is not directly related to their toxicity[11], it may play an important role in toxicological interactions such as the potentiation of toxicity of malathion by EPN inhibition of the AliE which is responsible for a major pathway of malathion degradation.[12] As shown by the data in Table 13, methamidophos is only a moderate inhibitor of house fly and bee abdomen aliesterases paralleling its anticholinesterase activity.

TABLE 13. INHIBITION OF ALIESTERASES FROM HOUSE FLY AND HONEY BEE ABDOMENS BY METHAMIDOPHOS.

	I_{50} (M)	
Substrate	House fly abdomens	Honey bee abdomens
Tri-n-butyrin	1.0×10^{-5}	2.8×10^{-5}
iso-Propyl propionate	-	2.3×10^{-2}
n-Butyl propionate	4.0×10^{-5}	$> 1 \times 10^{-2}$
iso-Butyl propionate	1.0×10^{-4}	-
n-Amyl propionate	8.0×10^{-5}	-
iso-Amyl propionate	1.6×10^{-5}	-

In Vivo Metabolism in the House Fly - The distribution of recovered radioactivity in various separated fractions from the susceptible NAIDM strain and resistant dimethoate strain of house flies at various times after treatment with ^{32}P-methamidophos is summarized in Tables 14 and 15. The amounts of radioactivity in the holding container rinses, which represent physically abraded and excreted materials, remained relatively constant with time and dosage for the NAIDM strain, suggesting that they are primarily abraded material. However, for the dimethoate strain, the amounts recovered in the holding container rinses increased with both time and dosage, suggesting that in the resistant strain appreciable amounts of methamidophos or metabolites were excreted in addition to those abraded. The radioactivity recovered in the fly rinse, which represents the materials remaining externally on the flies, decreased with time in both strains though at a faster rate in the NAIDM strain than in the dimethoate strain. Concomitantly, the radioactivity recovered in the fly extracts, which represents the extractable materials within the flies, and that in the fly unextractable residues, which represents the materials incorporated or bound to insoluble tissue residues, increased. The sum of the recovered radioactivity in the internal extracts and unextractable residues, which represents that portion of the dose which penetrated, also increased with time. The data for both external rinses and total penetration show that absorption was greater and faster in the susceptible NAIDM strain than in the resistant dimethoate strain, thus, at least in part, explaining the small but higher cross-resistance of the latter (10.7-fold) to methamidophos. This cross-resistance may also in part be attributable to greater excretion of methamidophos or metabolites in the resistant strain as noted above.

The various recovered fractions were analyzed for possible metabolites by paper chromatography and, after realkylation of potential ionic metabolites with diazomethane, by gas liquid chromatography. Only the

TABLE 14. DISTRIBUTION OF RECOVERED RADIOACTIVITY FROM THE NAIDM SUSCEPTIBLE STRAIN OF HOUSE FLIES TREATED TOPICALLY WITH ^{32}P-METHAMIDOPHOS

		µg of Applied Dose/Fly Recovered X 10^3				
Applied Dose µg/Female Fly (µg/g)	Time After Treatment Hour	Holding Container Rinse	Fly External Rinse	Fly Internal Extract	Fly Unextractable Residue	Total Penetration
0.012 (0.60)	0.5	0.9	5.1	5.1	.2	5.3
(12% mortality at 12 hr)	1	1.5	2.5	4.9	.3	5.2
	2	2.4	2.6	6.4	.6	7.0
	4	2.4	2.1	6.7	.8	7.5
	8	1.9	0.9	7.9	1.3	9.2
	12	1.8	0.8	8.1	1.3	9.4
	24	1.7	0.8	7.8	1.7	9.5
0.024 (1.2)	0.5	2.0	7.6	14	1.1	15.1
(10% mortality at 8 hr)	1	1.3	6.5	15	1.2	16.2
	2	1.3	4.5	16	2.0	18.0
	4	0.9	3.0	17	3.2	20.2
	8	1.6	1.4	16	3.4	19.4
0.05 (2.5)	0.5	3.3	26	18	2.1	20.1
(10% mortality at 4 hr)	1	2.6	24	20	3.1	23.1
	2	2.4	10	32	5.0	37.0
	4	2.8	6.1	34	7.7	41.7

TABLE 15. DISTRIBUTION OF RECOVERED RADIOACTIVITY FROM THE DIMETHOATE RESISTANT STRAIN OF HOUSE FLIES TREATED TOPICALLY WITH ^{32}P-METHAMIDOPHOS

		μg of Applied Dose/Fly Recovered X 10^3				
Applied Dose μg/Female Fly (μg/g)	Time After Treatment Hour	Holding Container Rinse	Fly External Rinse	Fly Internal Extract	Fly Unextractable Residue	Total Penetration
0.01 (0.50)	0.5	1.6	3.9	3.9	.6	4.5
(0% mortality at 24 hr)	1	1.2	4.6	3.6	.6	4.2
	2	1.3	3.5	4.3	.9	5.2
	4	1.7	2.7	4.6	1.1	5.7
	8	1.8	1.8	5.0	1.4	6.4
	12	1.8	1.7	5.1	1.4	6.5
	24	2.0	1.4	5.3	1.3	6.6
0.024 (1.2)	0.5	3.1	15.6	4.4	.6	5.0
(0% mortality at 4 hr)	1	3.5	13.4	6.3	.9	7.2
	2	4.6	10.4	6.2	3.1	9.3
	4	7.2	6.6	8.8	1.4	10.2
0.048 (2.4)	0.5	5.7	25	17	2.4	19.4
(0% mortality at 4 hr)	1	6.7	18	19	3.8	22.8
	2	7.4	15	20	6.1	26.1
	4	9.4	11	19	9.0	28.0
0.15 (7.5)	0.5	11	110	22	3.6	25.6
(5% mortality at 4 hr)	1	12	88	44	5.2	49.2
	2	22	76	43	7.6	50.6
	4	26	62	51	11	62

parent compound was detected in the holding container and external fly rinses and internal fly extracts. Fly-head ChE bioassay of the radioactively quantitated fractions showed no greater inhibition than would have been produced by similar amounts of methamidophos. Thus, no direct evidence for the in vivo conversion of methamidophos to either activation or degradation products was demonstrable.

In Vitro Metabolism - The results of incubating 1 X 10^{-3} M methamidophos with cockroach guts or mouse liver slices, followed by bioassay utilizing house fly head ChE inhibition, are shown in Table 16. The fact that inhibition after incubation essentially paralled that of the methamidophos standard suggests that methamidophos did not undergo any major activation or degradative changes in these systems as have previously been demonstrated for other organophosphorus compounds. However, compensating activities cannot be excluded on the basis of the bioassay results above.

Kinetics of Cholinesterase Inhibition - In order to develop further information on the inhibition of ChE by methamidophos, the kinetics of the inhibitory reaction were evaluated. The calculated affinity equilibrium constant (K_a), phosphorylation constant (k_p), and bimolecular inhibition constant (k_i) for methamidophos were respectively found to be: 5.5 X 10^{-4} (M), 1.3 (min^{-1}), and 3.3 X 10^{3} ($M^{-1}min^{-1}$). Since these values show that the affinity of methamidophos for ChE and its phosphorylation and bimolecular inhibition rates are all relatively low the amount of inhibited enzyme after short periods of incubation will be small. This explains the high I_{50} values (M) for methamidophos (Table 12), placing it at best in the class of a moderate cholinesterase inhibitor.

In Vivo Inhibition of Esterases - It is generally accepted that the toxic action of organophosphorus esters is due to their inhibition of acetylcholinesterase. In addition, there are other organophosphorus-sensitive esterases in house flies for which the term aliesterase was coined by van Asperen.[13] The results of the in vivo inhibition of ChE and AliE in house flies treated with methamidophos at the LD_{25}, LD_{50}, and LD_{90} levels are shown in Fig. 11, in which esterase activities of surviving flies are plotted against time after treatment. Knockdown and mortality were first evident nearly 8 hr after treatment at the LD_{25} and LD_{50} levels but occurred earlier at 4 hr at the LD_{90} level. ChE activities at these times were drastically reduced in knocked-down and dead flies to below 10% of normal activity in heads and to essentially 0% in thoraces. In surviving flies, ChE inhibition was slowly progressive and reached its maximum with the appearance of knockdown effects when head ChE activities at the LD_{25}, LD_{50}, and LD_{90} levels were respectively 64%, 16% and 16% of normal, and thoracic ChE activities were respectively 56%, 12% and 2%. Thus, the onset and severity of symptoms and degree of ChE inhibition in survivors were relatively dependent on the dosage level, in agreement with the results of a similar study with sarin.[14] There was little or no recovery of inhibited ChE with time in either the head or thoracic preparations of surviving flies treated with methamidophos.

TABLE 16. FLY HEAD ChE INHIBITION BY METHAMIDOPHOS AFTER SIX HOURS OF IN VITRO INCUBATION WITH COCKROACH GUTS OR MOUSE LIVER SLICES

Incubation System	Percent inhibition of fly-head ChE by aliquots of incubation extracts based on original concentration of methamidophos in incubation system					
	Molar Concentration					
	$1.1X10^{-6}$	$2.8X10^{-6}$	$5.6X10^{-6}$	$1.1X10^{-5}$	$5.5X10^{-5}$	$1.1X10^{-4}$
Cockroach Gut	15	27	36	60	88	95
Mouse liver Slices	20	10	20	34	56	96
Methamidophos Standard	14	29	40	50	78	92

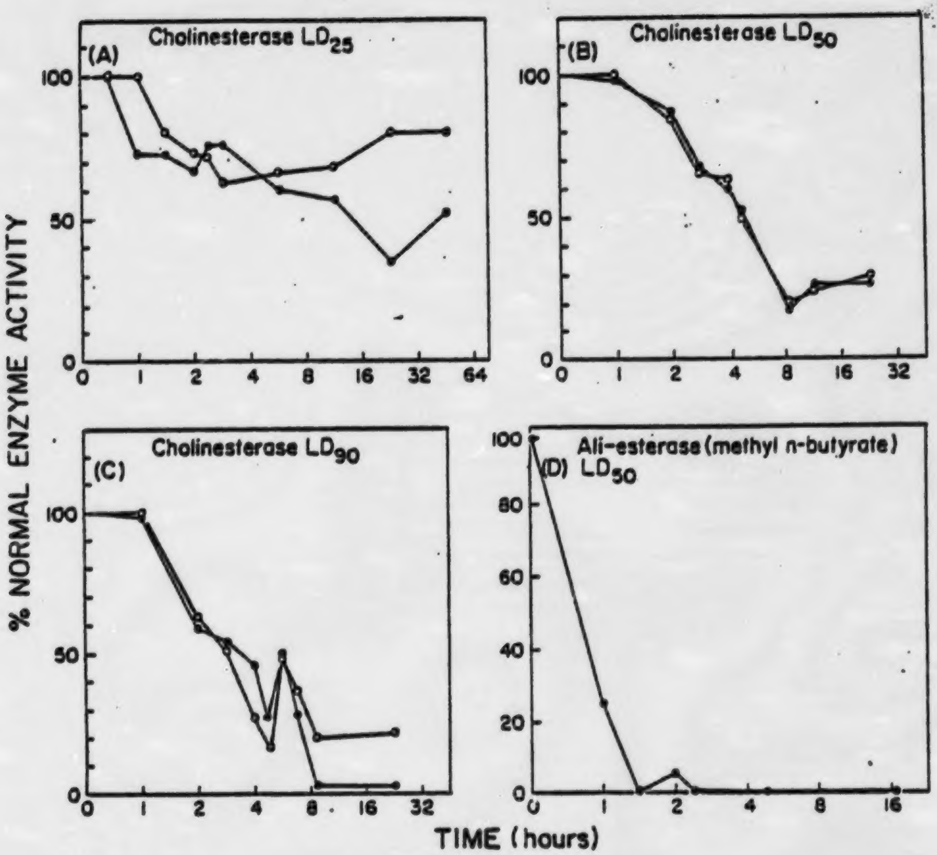


FIG. 11. Esterase activities in surviving NAIDM susceptible house flies treated on the tip of the abdomen with methamidophos at levels of the LD$_{25}$ (0.95 µg/g), LD$_{50}$ (1.2 µg/g), or LD$_{90}$ (2.0 µg/g). 0——0 head esterase; ●——● thoracic esterase.

Figure 11D shows that AliE inhibition after treatment with methamidophos at the LD_{50} level occurs sooner and is more complete in surviving flies than ChE inhibition, reaching essentially 0% of normal in 1.5 hr. AliE also does not recover over a period of 16 hr in the surviving flies. Since essentially total AliE inhibition occurred much earlier than the appearance of symptoms of poisoning, its inhibition by methamidophos appears to have little significance relative to poisoning effects, as has been similarly concluded for other organophosphorus compounds.[11]

Summary - Methamidophos is highly toxic to insects but at best is a moderate cholinesterase (ChE) inhibitor. Evaluation of the kinetics of its house fly ChE inhibition showed that its affinity for the enzyme and its phosphorylation and bimolecular inhibition rates are all relatively low. In vivo metabolism studies in house flies provided evidence that it is not activated to a more effective ChE inhibitor and indirect evidence also was obtained for its slow degradation. In vitro metabolism studies in house fly and mouse tissues provided additional evidence for its lack of activation and slow metabolic degradation. Compared to other effective organophosphorus insecticides, methamidophos was slow in producing acute symptoms of poisoning and ChE inhibition and required the accumulation of comparatively high internal levels for toxic effects. However, in vivo ChE inhibition studies provided evidence for the interrelationships of ChE inhibition and toxic effects. Thus, its relative stability and low in vivo degradation appeared to be of critical importance in accumulating and maintaining a sufficient internal concentration for a long enough period of time to permit the development of its slowly-expressed toxicity.

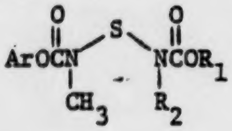
3. References.

1. McBain, J.B., I. Yamamoto and J.E. Casida, Life Sci. 10, 947-54 (1971).
2. McBain, J.B., L.J. Hoffman, J.J. Menn and J.E. Casida, Pestic. Biochem. Physiol. 1, 356-65 (1971).
3. Hoffman, L.J., J.M. Ford and J.J. Menn, Pestic. Biochem. Physiol. 1, 349-55 (1971).
4. McBain, J.B. and J.J. Menn, Biochem. Pharmacol. 18, 2282-85 (1969).
5. Ptashne, K.A. and R.A. Neal, J. Pharmacol. Exp. Ther. 179, 380-85 (1971).
6. Gillespie, R., J. Chem. Soc. 1002-13 (1952).
7. Kojima, K. and R.D. O'Brien, J. Agr. Food Chem. 16, 574-84 (1968).
8. Skrinjaric-Spoljar, M. and E. Reiner, Biochim. Biophys. Acta 165, 289-92 (1968).
9. Quistad, G.B., T.R. Fukuto and R.L. Metcalf, J. Agr. Food Chem. 18, 189 (1970).
10. van Asperen, K., Biochem. Pharmacol. 3, 136 (1960).
11. Stegwee, D., Nature 184, 1253 (1959).
12. DuBois, K.P., F.K. Kinoshita and J.P. Frawley, Toxicol. Appl. Pharmacol. 12, 273 (1968).
13. van Asperen, K., Nature 181, 355 (1958).
14. Mengle, D.C. and J.E. Casida, J. Econ. Entomol. 51, 750 (1958).

B. INSECTICIDE SELECTIVITY

1. Selective Toxicity of *N,N'*-Thiodicarbamates.

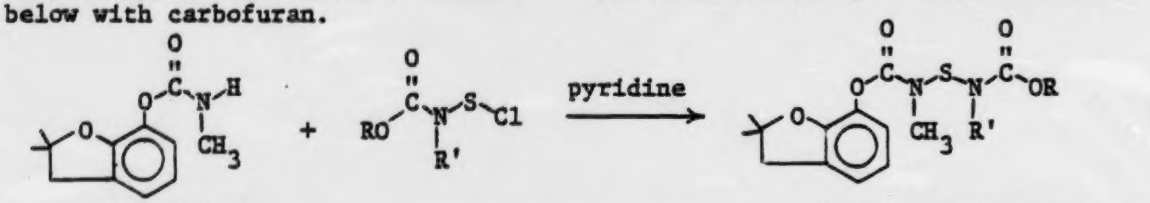
Introduction - In earlier work, the favorable toxicological properties of a series of N-substituted bis-carbamoyl sulfides was revealed. These bis-carbamoyl sulfide derivatives of methylcarbamate insecticides still retained the good insecticidal activity of the parent methylcarbamate but were substantially less toxic to the white mouse. Because of the desired order of selectivity demonstrated by these bis-carbamoyl sulfides, it was of interest to examine other derivatives of this type for selective toxicity. This section is concerned with the synthesis and toxicological properties of a series of unsymmetrical N,N'-thiodicarbamate derivatives of the general structure shown below,



$$\mathrm{ArOC(=O)N(CH_3){-}S{-}N(R_2)C(=O)OR_1}$$

where Ar is the phenolic moiety of carbofuran, propoxur, carbaryl, m-isopropylphenyl methylcarbamate or oxime of aldicarb, methomyl, and oxamyl, and R_1 and R_2 are aliphatic groups.

Synthesis of *N,N'*-Thiodicarbamates - These derivatives of insecticidal methylcarbamates were synthesized by the reaction between the methylcarbamate and alkyl alkyl(chlorosulfenyl)carbamate in pyridine according to the equation below with carbofuran.



(carbofuran)

(N,N'-thiodicarbamate)

Toxicological Properties - Data for the toxicity of the various N,N'-thiodicarbamates to house flies, mosquito larvae, and white mice (oral) are given in Table 1. In order to account for differences in molecular weights of the compounds, toxicity data also are expressed on a mole or molar basis as well as on a weight basis, i.e., LD_{50} is given in terms of μmole/kg (mouse) and LD_{50} to mosquito larvae is given as μM solution.

Toxicity to House Flies - Examination of the house fly toxicity data reveals that on a mole/g basis the toxicity of the N,N'-thiodicarbamates to house flies were generally equal to the toxicity of the parent methylcarbamate. For example, in the carbofuran series (1-12) toxicity ranged from 0.023 to 0.031 μmole/g and no discernible trend in toxicity was observed with change in structure. The virtually identical toxicities of these compounds to house flies was somewhat surprising owing to the large change in molecular weights (>2-fold range) of the derivatives and commensurate change in physical

TABLE 1. TOXICITY OF N,N'-THIODICARBAMATES TO HOUSE FLIES, MOSQUITO LARVAE, AND MICE.

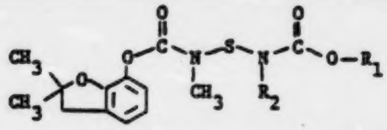
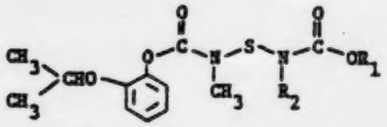
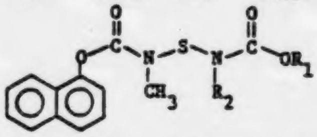
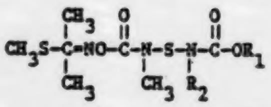
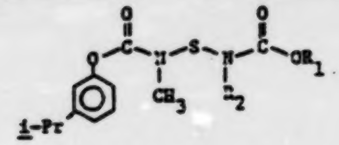
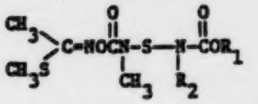
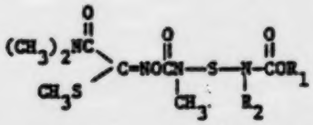
No.	R_1	R_2	MW	House fly LD_{50}		*Culex pipiens* LC_{50}		Mouse (oral) LD_{50}	
				µg/g	µmole/g	ppm	µM	mg/kg	µmole/kg
									
Carbofuran	-	-	221.3	6.70	0.030	0.052	0.235	2	9
1			340.3	9.25	0.027	0.023	0.0676	45	132
2	Et	Me	354.4	9.0	0.025	0.016	0.0452	72	203
3	n-Pr	Me	368.5	8.5	0.023	0.009	0.0245	88	238
4	i-Pr	Me	368.5	9.0	0.024	0.0088	0.0239	52	141
5	n-Bu	Me	382.5	10.0	0.026	0.0043	0.0113	130	340
6	C_5H_{11}	Me	396.5	10.5	0.027	0.0022	0.0056	140	353
7	C_7H_{15}	Me	424.6	11.0	0.026	0.0010	0.0024	175	412
8	C_8H_{17}	Me	438.6	12.0	0.027	0.0008	0.0019	190	433
9	$C_{10}H_{21}$	Me	466.6	14.0	0.030	0.0012	0.0026	250	536
10	Me	Et	354.4	9.0	0.025	0.0160	0.0452	62	175
11	Et	Et	368.5	9.25	0.025	0.0100	0.0272	85	231
12	Et	i-Pr	382.5	12.0	0.031	0.0056	0.0147	125	327
									
Propoxur	-	-	209.2	24	0.115	0.33	1.577	62	-
13	Et	Et	356.4	37.5	0.105	0.046	0.129	>1000	-
14	C_5H_{11}	Me	384.5	35.0	0.091	0.008	0.021	>1000	-
15	C_7H_{15}	Me	412.5	44.0	0.107	0.0008	0.002	>3000	-
16	$C_{10}H_{21}$	Me	454.6	44.0	0.097	0.009	0.020	>1000	-
									
Carbaryl	-	-	201.2	900[a]	4.473	1.0	4.967	500[b]	-
17	C_8H_{17}	Me	418.5	350	0.836	0.08	0.191	1000	-
 $CH_3S-\underset{CH_3}{\overset{CH_3}{C}}=NO-\overset{O}{\overset{\Vert}{C}}-\underset{CH_3}{N}-S-\underset{R_2}{N}-\overset{O}{\overset{\Vert}{C}}-OR_1$									
Aldicarb	-	-	190.2	5.5	0.029	0.16	0.841	0.4	2.1
18	$C_{10}H_{21}$	Me	435.5	13.0	0.030	0.0064	0.015	12.5	28.7

TABLE 1. (cont'd)

No.	R_1	R_2	MW	House fly LD_{50} µg/g	House fly LD_{50} µmole/g	*Culex pipiens* LC_{50} ppm	*Culex pipiens* LC_{50} µM	Mouse (oral) LD_{50} mg/kg	Mouse (oral) LD_{50} µmole/kg
									
MIP	-	-	193.2	41	0.207	0.038	0.197	16	83
19	Me	Me	312.4	80	0.256	0.015	0.048	86	275
20	*n*-Pr	Me	340.4	75	0.220	0.006	0.018	195	573
21	*i*-Pr	Me	340.4	72.5	0.213	0.0056	0.017	200	588
22	*i*-Pr	Et	354.4	62.5	0.176	0.000078	0.00022	340	959
23	*n*-Bu	Me	354.4	80	0.226	0.0001	0.00028	310	875
24	C_7H_{15}	Me	396.6	87.5	0.221	0.000068	0.00017	530	1336
									
Methomyl	-	-	162	3.7	0.023	0.64	3.95	10	62
25	Me	Me	281.4	7.0	0.025	0.92	3.27	160	569
26	Et	Me	295.4	7.3	0.025	0.70	2.37	310	1049
27	*n*-Pr	Me	309.4	8.8	0.028	0.68	2.19	400	1293
28	*i*-Pr	Me	309.4	8.5	0.028	0.70	2.26	345	1115
29	*i*-Pr	Et	323.4	10.5	0.033	0.64	1.98	450	1392
30	C_6H_{13}	Et	365.5	7.5	0.021	0.08	0.22	600	1642
									
Oxamyl	-	-	219	3.6	0.016	0.33	1.51	5[b]	23
31	Me	Me	338.4	3.2	0.010	0.37	1.09	9	27
32	Et	Me	352.4	3.7	0.011	0.41	1.16	10	28
33	*i*-Pr	Me	366.5	4.2	0.012	0.37	1.01	10	27
34	*i*-Pr	Et	380.5	4.5	0.012	0.32	0.84	9	24
35	C_6H_{13}	Et	422.6	6.0	0.014	0.08	0.19	12	28

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[a] extrapolated value; [b] value obtained on rats

properties. For example, the octanol-water partition coefficient P varied from 191 for 1 to 6 X 10^6 for 9. The difference in hydrophobic character between 1 and 9 is undoubtedly very large and, therefore, a large difference in the ability of these compounds to penetrate into the house fly was expected. Also, one would expect a large difference in penetration between water-soluble carbamates such as methomyl and oxamyl and their respective lipophilic derivatives, e.g., 30 and 35.

Assuming that *in vivo* release of the toxic parent methylcarbamate is responsible for the toxicity of the *N*,*N*'-thiodicarbamate, e.g., carbofuran from compounds 1-12, the nearly equal toxicities observed within a homologous series suggests that nearly equal amounts of parent methylcarbamate are produced from each derivative. Further, since the toxicity of the derivatives are equal or possibly slightly greater than the parent methylcarbamate on a mole basis, it appears that conversion of the derivative to the methylcarbamate is rapid.

Toxicity to Mosquito Larvae — The data in Table 1 show that, in contrast to house flies, toxicity to mosquito larvae systematically increased with increase in carbon atoms in the aliphatic carbamate moiety. Maximum toxicity was observed when the total number of carbon atoms was eight or nine and further increase in carbons resulted in a gradual decrease in toxicity. These results suggest that hydrophobicity of the molecule plays an important role in mosquito larvae intoxication by the derivatized methylcarbamates. In the aqueous habitat of the larvae, the carbamate derivative must partition between water and the hydrophobic epicuticular wax layer of the larvae and derivatives of greater hydrophobicity should move more readily into the larval cuticle. However, when the number of carbon atoms becomes too large, the molecules become too insoluble in water and either are present in aggregates which are less available to the larvae or are trapped in the larval wax, resulting in reduced subsequent movement.

Figure 1 provides a graphic relationship between the logarithm of the toxicity of the various sulfenylated derivatives of carbofuran (log LC_{50}, μmolar) and logarithm of the octanol-water partition coefficients (log P). The circles represent actual values of log LC_{50} and log P for each compound and the solid line is the best parabolic fit of the points according to the equation 1, obtained by multiple regression analysis.

$$\log LC_{50} = 1.24 - 1.19 \log P + 0.09(\log P)^2 \qquad (1)$$

$$\underline{n} = 12 \qquad \underline{r} = 0.988$$

n is the number of compounds and *r* is the multiple linear regression coefficient. Neither the position of the alkyl chain, i.e., on nitrogen or oxygen, nor branching had any effect on the correlation. A similar relationship between log P and mosquito larvicidal toxicity was observed for other *N*,*N*'-thiodicarbamates such as the homologous series of propoxur (13-16), MIP (19-24), methomyl (25-30), and oxamyl derivatives (31-35).

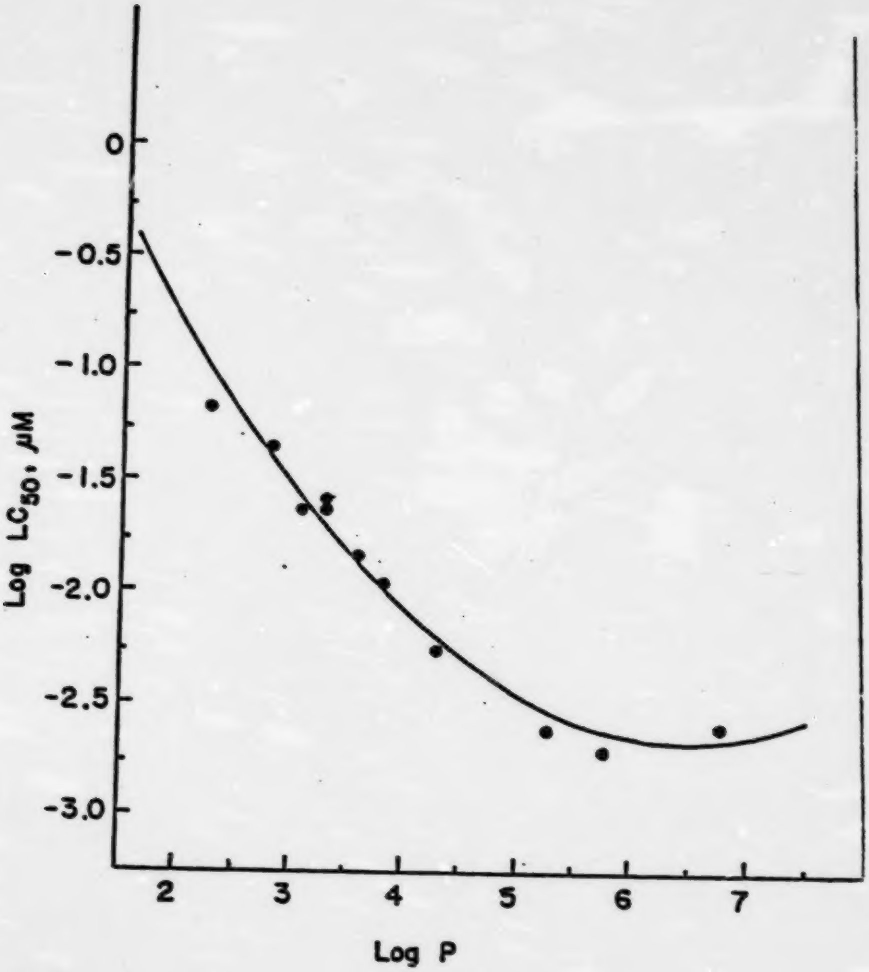
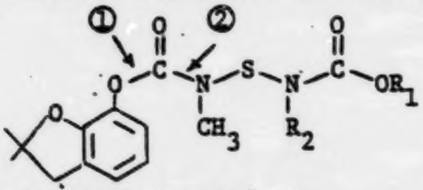


FIG. 1. Relationship between toxicity of sulfenyl carbofuran derivatives to mosquito larvae (log LC_{50}, μM) and logarithm of the octanol-water partition coefficient (log P).

Compared to propoxur and MIP, two of their sulfenylated derivatives **15** and **24**, were astonishingly effective against mosquito larvae. Compound **15** or *n*-heptyl methylcarbamylosulfenyl derivative of propoxur is almost 800-fold more toxic to mosquito larvae than propoxur and **24** is almost 1200-fold more toxic than MIP. The LC_{50} value of 0.000068 ppm for **24** makes it one of the most effective mosquito larvicides ever tested in this laboratory.

Toxicity to Mice - LD_{50} values for the toxicity of the *N*,*N*'-thiodicarbamates to mice are given in Table 1 in terms of both mg/kg and μmole/kg. With the exception of the oxamyl derivatives, the results clearly indicate that, in addition to their high insecticidal activity, the *N*,*N*'-thiodicarbamates are remarkably less toxic to the white mouse than the parent methylcarbamate. For example, on a μmole basis, **9** was 60-fold less toxic to the white mouse than carbofuran. In fact, **7**, **8**, and **9** were safer to mice than any other sulfenylated derivative of carbofuran previously examined in this laboratory[1,2,3]. Derivatization of aldicarb resulted in a thiodicarbamate (**18**) which was significantly less toxic to the mouse than aldicarb, but because of the exceptionally high toxicity of aldicarb, **18** was still undesirably toxic.

Earlier studies[4,5] on the metabolism and mode of action of derivatized methylcarbamate esters have shown that their low mammalian toxicity is attributable to preferential metabolic detoxication of the derivative to nontoxic phenols. It is reasonable that this also is the explanation for the mammalian safety of the *N*,*N*'-thiodicarbamates. Cleavage at points 1 or 2 in the structure below



would result in nontoxic products.

As in the case of mosquito larvicidal activity, mouse toxicity also appeared to be dependent on the hydrophobic character of the derivative. Figure 2 shows the relation between mice toxicity (LD_{50} μmole/kg) and log P for the homologous series of carbofuran derivatives (**1**-**12**). Evidently, toxicity decreases with increasing log P in two distinct linear relationships. By means of linear regression analysis, equations 2 and 3 were computed to give best fit to the data.

$$\log LD_{50} = 1.503 + 0.272 \log P \qquad r = 0.968 \qquad (2)$$

$$\log LD_{50} = 2.273 + 0.066 \log P \qquad r = 0.994 \qquad (3)$$

Log P for the point at which the two lines intersect was calculated to be 3.74; this corresponds to a P value of 5500. While the biphasic nature of the relationship in Fig. 2 is not understood, it raises the possibility of a change-over in intoxication process which depends upon the hydrophobic

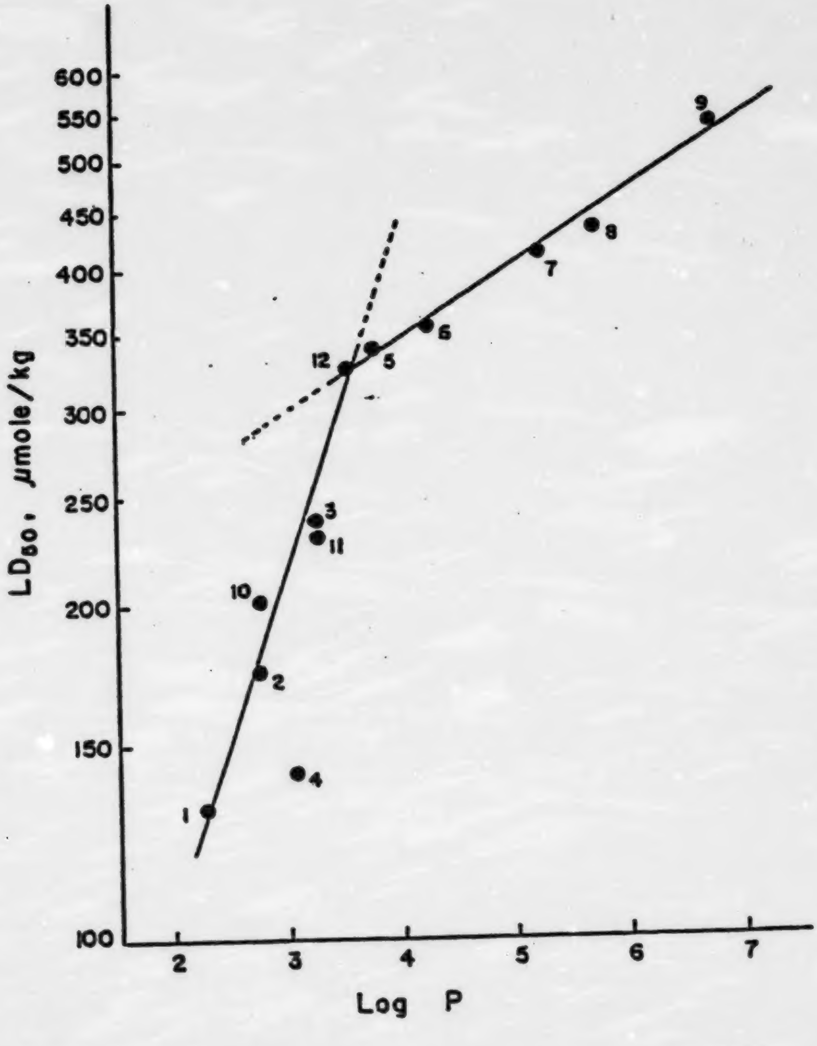


FIG. 2. Relationship between toxicity of sulfenyl carbofuran derivatives to white mice (log LD_{50}, μmole/kg) and logarithm of the octanol-water partition coefficient (log P).

properties of the molecule. This biphasic relationship between mammalian toxicity and log P also was evident for the other carbamates such as the MIP derivatives 19-24 and methomyl derivatives 25-30, and therefore, may be of a general nature.

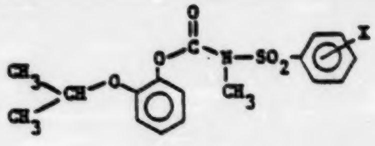
Summary - A series of N-(alkyl alkylcarbamylosulfenyl) derivatives of the methylcarbamate insecticides carbofuran, m-isopropylphenyl methylcarbamate, propoxur, aldicarb, methomyl and oxamyl were prepared and examined for toxicity to house flies, mosquito larvae, and white mice. Compared to the parent methylcarbamate, the derivatives were generally of equal toxicity to the house fly and substantially more toxic to mosquito larvae. With the exception of the oxamyl derivatives, all of the derivatives were much less acutely toxic to the white mouse. The toxicities of the derivatives to mosquito larvae and white mice were correlated with their octanol-water partition coefficients.

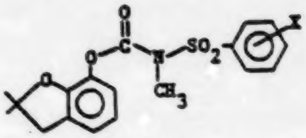
2. Insecticidal Properties of the N-Sulfonyl Derivatives of Propoxur and Carbofuran.

Introduction - Derivatization of the carbamate moiety of toxic methylcarbamate insecticides generally results in compounds of low mammalian toxicity and high insecticidal activity[1,2,3]. While a wide variety of sulfenyl derivatives of methylcarbamate insecticides have been examined for toxicological properties, little has been reported on the insecticidal activity of sulfur derivatives of higher oxidation state. In a previous report[6], we described the poor insecticidal activity of a single arylsulfonyl derivative, the N-2-toluenesulfonyl derivative of carbofuran. Work was conducted to determine whether derivatization with substituted benzenesulfonyl groups containing electron-withdrawing moieties would result in compounds with higher insecticidal activity.

Toxicological Properties - Toxicological data are presented in Table 2. Compared to the N-arylsulfenyl derivatives of carbofuran and propoxur, the substituted benzenesulfonyl derivatives were relatively ineffective against house flies and mosquito larvae. In fact, none of the sulfonyl derivatives were effective against house flies at 500 µg/g and cotreatment with 5 parts of piperonyl butoxide was required before any sign of intoxication was evident. The poor insecticidal activity of the substituted benzenesulfonyl derivatives listed in Table 1 suggest that the parent methylcarbamates are not readily generated from the sulfonyl derivatives in insects. If it is assumed that the parent methylcarbamate is responsible for intoxication, then the synergized house fly toxicity of compounds 42 and 49 suggests that generation of the methylcarbamate takes place to a greater extent when two strongly electron-withdrawing nitro groups are attached to the benzenesulfonyl ring. In these cases, the two nitro groups probably promote hydrolytic cleavage of the nitrogen-sulfur bond, resulting in some formation of the parent methylcarbamate in vivo, at least to the extent that intoxication of house flies is observed when a synergist is present.

TABLE 2. INSECTICIDAL PROPERTIES OF SUBSTITUTED BENZENESULFONYL DERIVATIVES OF PROPOXUR AND CARBOFURAN.



No.	X	House fly LD$_{50}$, µg/g		Culex fatigans LC$_{50}$, ppm
		alone	1:5 P.B.	
36	4-Cl	>500	>50	>10
37	4-Br	>500	>50	>10
38	2-NO$_2$	>500	50	6.8
39	3-NO$_2$	>500	>50	>10
40	4-NO$_2$	>500	>50	7.5
41	2,5-di-Cl	>500	>50	4.2
42	2,4-di-NO$_2$	>500	16	0.17
				
43	4-Cl	>500	>50	>10
44	4-Br	>500	>50	>10
45	2-NO$_2$	>500	20	0.26
46	3-NO$_2$	>500	>50	4.0
47	4-NO$_2$	>500	>50	0.14
48	2,5-di-Cl	>500	>50	>10
49	2,4-di-NO$_2$	>500	9	0.11

Qualitative examination of 49 showed that it is not easily converted to carbofuran and is transformed into a variety of products in the presence of different reagents. Compound 49 was stable in acetone-water solution and returned virtually all of the starting material after standing for 70 hr at room temperature. In the presence of either a sulfhydral-containing agent (thiocresylate) or base (triethylamine), 49 decomposed to a variety of compounds but did not produce carbofuran.

Compounds 43 and 49 were examined for their toxicity to the white mouse. Neither compound produced any symptoms of intoxication in mice treated at 400 mg/kg. As a reference point, the LD$_{50}$ value of carbofuran to mice is 10 mg/kg. Therefore, substituted benzenesulfonyl derivatives of toxic methylcarbamates are neither toxic to insects nor mammals.

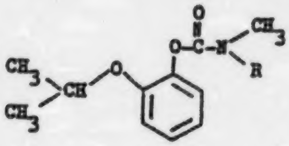
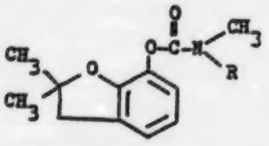
Summary - A series of substituted N-benzenesulfonyl derivatives of carbofuran and propoxur containing electron-withdrawing substituents on the benzene ring were synthesized and examined for toxicity to house flies and mosquito larvae. In general, the derivatives were noninsecticidal when tested alone, but some of the compounds were toxic to house flies when synergized with piperonyl butoxide. The poor insecticidal activity and low mammalian toxicity of these derivatives is attributable to their high stability and their inability to be converted into the parent methylcarbamate.

3. Selective Toxicity of *N*-Arylsulfenyl Derivatives of Methylcarbamate Insecticides.

Introduction - Less emphasis has been placed in past years on studies concerned with differences in toxicity of insecticidal materials toward different insect species, particularly with regard to differences in response between beneficial and harmful insects. Although organophosphorus esters containing the diisopropoxy moiety, e.g., diisopropyl parathion, are known to be peculiarly safe to the honey bee while still retaining high activity towards other insects[7,8], carbamate insecticides generally are considered to be highly toxic to the honey bee. Because of the favorable order of selectivity achieved between insects and mammals by derivatization of carbamate insecticides, an assessment of the toxicity of derivatized methylcarbamates to the honey bee also was made. This section is concerned with the toxicological properties of a series of N-sulfenylated derivatives of commercial methylcarbamate insecticides with special focus on the toxicological response observed in honey bees. Work on the comparative metabolism of one of the derivatives was conducted in the honey bee and house fly to determine the basis for its selective toxicity.

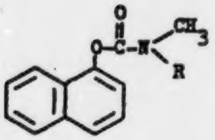
Insecticidal Activity - Data for the toxicity of the various N-arylsulfenylated derivatives of insecticidal methylcarbamates to honey bees and house flies are given in Table 3. Toxicity ratios, defined as LD_{50} of the compound against the honey bee divided by the LD_{50} of the compound against the house fly, also are given. It is apparent from the data in Table 3 that most of the various substituted phenylsulfenylated derivatives of propoxur and carbofuran were highly toxic to the honey bee. In the propoxur series, very little difference was observed between the toxicity ratios of propoxur and sulfenylated derivatives 50, 51, 52 and 55 with toxicity ratios ranging from 0.19 to 0.29. Slight improvement in bee toxicity was observed with the bromine containing derivatives (58, 59) and for the 2-tolyl-(53) and 2,4-xylysulfenyl (54) derivatives, but all of these compounds were substantially more toxic to the honey bee than to the house fly. Introduction of a t-butyl moiety in the phenylsulfenyl ring resulted in a derivative (56) which was 4-fold less toxic to the honey bee than to the house fly and was 8-fold less toxic to the honey bee than propoxur. Further insertion of a methyl moiety in the 2-position of the phenyl group, giving the 2-methyl-4-t-butylphenylsulfenyl derivative (57), resulted in a compound which was virtually nontoxic to the honey bee while remaining as effective as propoxur to the house fly. Thus, the introduction of a single methyl group in VIII increased bee safety more than 8-fold.

TABLE 3. TOXICITY OF N-ARYLSULFENYL AND N-ALKYLSULFENYL DERIVATIVES OF INSECTICIDAL METHYLCARBAMATES TO HOUSE FLIES AND HONEY BEES

Compd	R	House Fly LD_{50} μg/g	Honey Bee LD_{50} μg/g	Toxicity Ratio
				
	H(Propoxur)	24.0	4.5	0.19
50	Phenylsulfenyl	36.0	6.4	0.18
51	4-Tolylsulfenyl	36.0	8.0	0.22
52	3-Tolylsulfenyl	23.5	6.9	0.29
53	2-Tolylsulfenyl	24.0	10.0	0.42
54	2,4-Xylylsulfenyl	27.5	16.8	0.61
55	2-Isopropylphenylsulfenyl	28.5	5.4	0.19
56	4-t-Butylphenylsulfenyl	9.0	36.0	4.00
57	2-Me-4-t-butylphenylsulfenyl	24.5	>800.0	>32.70
58	4-Br-Phenylsulfenyl	26.5	14.8	0.52
59	4-Br-2-Me-Phenylsulfenyl	37.0	15.0	0.41
60	CH_3	65.0	3.0	0.05
				
61	H(Carbofuran)	6.7	0.42	0.06
62	Phenylsulfenyl	9.3	0.52	0.05
63	4-Tolylsulfenyl	9.0	0.72	0.08

continued next page

TABLE 3. (cont'd)

Compd	R	House Fly LD$_{50}$ μg/g	Honey Bee LD$_{50}$ μg/g	Toxicity Ratio
64	3-Tolylsulfenyl	6.5	0.66	0.10
65	2-Tolylsulfenyl	3.7	0.52	0.14
66	4-*t*-Butylphenylsulfenyl	2.7	1.42	0.53
67	2-Me-4-*t*-butylphenylsulfenyl	7.5	2.34	0.31
68	4-Br-phenylsulfenyl	9.0	0.80	0.09
69	4-Br-2-Me-phenylsulfenyl	11.3	0.8 to 4.0	-
70	2-Isopropylphenylsulfenyl	8.3	0.8 to 4.0	-
				
71	H(Carbaryl)	>900.0	18.4	<0.02
72	2-Me-4-*t*-butylphenylsulfenyl	>500.0	>800.0	>1.6

In contrast to the propoxur derivative, the corresponding substituted phenylsulfenyl derivatives of carbofuran were all more toxic to the honey bee than to the house fly, including the 4-t-butyl- and 2-methyl-4-t-butylphenylsulfenyl analogs. However, conversion of carbaryl to the 2-methyl-4-t-butylphenylsulfenyl derivative resulted in a compound which was more than 44-fold less toxic to the honey bee than carbaryl (compare 71 with 72).

In the presence of piperonyl butoxide, an inhibitor of mixed-function oxidase enzyme activity, the toxicity of 57 to honey bees was significantly increased (LD_{50} 44·0 µg/g). The high degree of synergism obtained with piperonyl butoxide probably resides in the ability of the synergist to protect the methylcarbamate from detoxication by mixed-function oxidase enzymes after its formation from the derivative. The high toxicity of 57 in combination with piperonyl butoxide to bees clearly suggests a significant role for mixed-function oxidase enzymes in bees.

Metabolism Studies - Because of the unusual safety of the 2-methyl-4-t-butylphenylsulfenyl derivative (57) of propoxur to the honey bee and its high toxicity to the house fly, work on the comparative metabolism of 57 was conducted to determine the basis for selectivity.

Control Experiments - During a preliminary metabolism study of 57 in the honey bee, large amounts of propoxur were detected in the internal extract of the bee. This finding was disturbing since propoxur is highly toxic to the bee. Therefore, the effect of biological tissue on the generation of propoxur from 57 through N-S bond cleavage during the work-up procedure for metabolite isolation was investigated. Data for the formation of propoxur upon incubation of 57 with honey bee homogenate with and without N-ethylmaleimide (NEM), a well-known sulfhydryl inhibitor, are presented in Table 4. The use of NEM was suggested from earlier studies on the effect of NEM on animal tissue-catalyzed generation of carbofuran from N-(2-toluenesulfenyl)-carbofuran[9]. In the control experiment without NEM, 55% of the applied dose was recovered as 57 and the rest as propoxur. This indicates that 57 was being converted to propoxur in the tissue homogenate during the extraction procedure. In the presence of NEM, the production of propoxur was markedly inhibited and sulfenyl-propoxur was recovered in large quantities. Workup of honey bees and house flies treated with $[^{14}C]$57, therefore, was carried out by adding 15 ml 10^{-2}M NEM at the time of homogenation.

Penetration - Data for the penetration of 57 into honey bees and house flies are presented in Table 5. Values are expressed in terms of total external and internal recoveries of applied radioactivity. Radioactivity collected from the insect body wash and cage rinse was designated external recovery and that collect from the insect homogenate and tissue residue was designated internal recovery.

TABLE 4. STABILITY OF THE [^{14}C]SULFENYL-PROPOXUR DURING METABOLITES EXTRACTION WORKUP PROCEDURE IN HONEY BEE HOMOGENATE.

Dose	Recovery of the applied dose			
	sulfenyl-propoxur		propoxur	
	%	μg/g	%	μg/g
1. Control, 16 μg/g	55.0	8.80	45.0	3.84
2. N-Ethylmaleimide (NEM), 10^{-2}M in tissue preparation[a]				
a. 23.71 μg/g	98.8	23.43	1.2	0.14
b. 300.00 μg/g	99.4	298.32	0.6	0.89

[a] Honey bee homogenate preparation was incubated with the indicated concentration of NEM for 15 min prior to reaction with 57.

At the lower dosage of 24.5 μg/g, penetration of 57 into the honey bee was significantly slower than penetration into the house fly. This is evident from examination of either the external or internal recovery data. For example, based on internal recoveries about 2.5-fold more radioactivity was present in the house fly than in the honey bee 1 hr following treatment. While direct comparisons cannot be made, it is apparent that the differences in amounts penetrated decreased with time; however, noticeably more radioactivity was present in flies after 12 hr than in bees after 18 hr.

At the higher dosage of 300 μg/g a smaller fraction of the applied amount penetrated into the bee than at 24.5 μg/g. However, owing to the much larger amount of 57 applied, substantially greater quantities of radioactivity were present in the bee at the higher dosage. For example, 1 hr after treatment 6.7-fold more radioactivity was detected internally in bees treated at the higher dosage. Since 12-fold more 57 was applied at 300 μg/g relative to 24.5 μg/g, the smaller difference in the amount penetrated suggests that 300 μg/g exceeds the saturation dosage.

Compared to 57, substantially larger percentages of propoxur were absorbed by the honey bee. However, because of the large difference in the amounts applied, i.e., 24.5 μg/g vs 4.5 μg/g, direct comparisons cannot be made. It should be pointed out that in terms of actual weight, about the same amount of radioactivity was present internally in bees 4 hr after treatment with either 24.5 μg/g 57 or 4.5 μg/g propoxur (about 2.8 μg/g).

Metabolism in Honey Bees and House Flies Treated With 24.5 μg/g 57 - Data indicating the nature and distribution of metabolites recovered and identified from honey bees and house flies treated with 24.5 μg/g 57 are presented in Tables 6 and 7. Four principal metabolites, identified as propoxur, N-CH_2OH-propoxur, O-de-i-propyl-propoxur, and 5-hydroxy-propoxur, along with large amounts of the starting sulfenyl-propoxur were isolated from both honey bees and house flies.

TABLE 5. TOTAL EXTERNAL AND INTERNAL RECOVERY OF APPLIED RADIOACTIVITY FROM HONEY BEES AND HOUSE FLIES TREATED WITH COMPOUNDS AT VARIOUS TIME INTERVALS

					[^{14}C] Recovered			
					Total External (cage rinse + body rinse)		Total Internal (homogenate extract + residue)	
Insect	Compound	Dose (μg/g)	Time (hours)	Number of insects used	%	μg/g	%	μg/g
Honey bee	sulfenyl-propoxur	24.5	1	70	88.9	21.8	8.4	2.1
			4	70	80.6	19.7	11.4	2.8
			8	80	77.7	19.0	18.4	4.5
			18	59	66.7	16.3	30.7	7.5
House fly	sulfenyl-propoxur	24.5	1	140	75.7	18.5	21.4	5.2
			12	228	50.0	12.3	48.1	11.8
Honey bee	sulfenyl-propoxur	300.0	1	30	89.3	267.9	4.6	13.8
			4	35	92.3	276.9	5.6	16.8
Honey bee	propoxur	4.5	1	40	62.6	2.8	36.8	1.7
			4	23	36.7	1.7	60.3	2.7

TABLE 6. DISTRIBUTION AND IDENTIFICATION OF METABOLIC PRODUCTS RECOVERED FROM THE HONEY BEE AFTER TOPICAL APPLICATION OF 24.5 µg/g OF SULFENYL-PROPOXUR AT VARIOUS TIME INTERVALS

	1 hr		4 hr		8 hr		18 hr	
	%	µg/g	%	µg/g	%	µg/g	%	µg/g
Cage Rinse								
sulfenyl-propoxur	2.6	0.64	8.6	2.1	22.2	5.43	14.2	3.49
propoxur	-	-	-	-	0.3	0.04	0.8	0.10
N-CH$_2$OH-propoxur	-	-	-	-	0.1	0.01	0.2	0.02
O-de-*i*-propyl-propoxur	-	-	-	-	-	-	0.1	0.01
5-hydroxy-propoxur	-	-	-	-	-	-	-	-
others	0.1	-	0.3	-	1.2	-	0.3	-
unextractables	0.6	-	-	-	0.4	-	0.3	-
Total	3.3	-	8.9	-	24.2	-	15.9	-
Body Rinse								
sulfenyl-propoxur	84.4	20.68	70.2	17.21	53.1	13.01	46.9	11.48
propoxur	0.4	0.05	-	-	0.1	0.01	1.0	0.16
others	0.7	-	1.5	-	0.4	-	2.8	-
Total	85.5	-	71.7	-	53.6	-	50.7	-
Internal Extracts								
Organosoluble extracts								
sulfenyl-propoxur	3.1	0.76	1.9	0.46	3.4	0.84	6.8	1.68
propoxur	0.9	0.11	1.5	0.19	1.5	0.20	2.1	0.27
N-CH$_2$OH-propoxur	0.1	0.01	2.6	0.36	4.6	0.65	5.8	0.82
O-de-*i*-propyl-propoxur	-	-	1.0	0.13	2.8	0.29	4.4	0.46
5-hydroxy-propoxur	-	-	0.3	0.04	0.3	0.04	0.7	0.10
others	-	-	0.3	-	1.1	-	0.6	-
Conjugates								
sulfenyl-propoxur	1.0	0.25	0.1	0.02	0.1	0.01	0.2	0.05
propoxur	-	-	0.1	0.01	-	-	0.2	0.03
N-CH$_2$OH-propoxur	0.1	0.01	0.2	0.03	0.1	0.02	1.6	0.23
O-de-*i*-propyl-propoxur	-	-	0.4	0.04	-	-	0.9	0.10
others	0.1	-	0.1	-	-	-	0.2	-
unextractables	2.0	-	1.0	-	2.3	-	4.9	-
Total	7.5	-	9.5	-	16.2	-	28.6	-
Residue	1.1	-	1.7	-	2.2	-	2.0	-
Total radioactivity Recovered	97.4		91.8		96.2		97.2	

Assuming that propoxur is the metabolite responsible for the toxicity of 57, it is of interest to compare the levels of propoxur recovered from the internal extracts of the honey bee and house fly. From the data in Table 6, propoxur evidently is maintained at relatively low levels in the honey bee. The relative amounts of propoxur along with 57 and the other metabolites in the honey bee at increasing time intervals are depicted graphically in Fig. 3. The figure shows a steady level of internal propoxur and increasing amounts of hydroxylated products with time. At all time intervals the parent compound 57 remained the major internal radioactive constituent and, after an initial drop, this material increased at a faster rate than the other products. The results suggest a slow, steady conversion of 57 to propoxur which, in turn, is detoxified to hydroxylated products as it is formed. In this manner propoxur maintains a steady state level and does not accumulate to levels needed for intoxication of the bee.

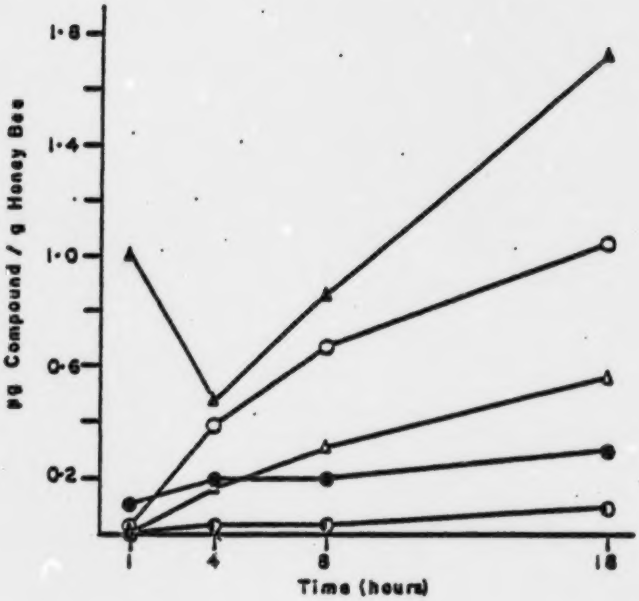


FIG. 3. Internally recovered metabolites from the honey bee after topical application of 24.5 µg/g of sulfenyl-propoxur at various time intervals. (▲—▲) sulfenyl-propoxur; (●---●) propoxur; (O---O) *N*-CH$_2$OH-propoxur; (△---△) *O*-de-*i*-propyl-propoxur; (◐---◐) 5-hydroxy-propoxur.

Examination of the house fly data (Table 7) shows substantially larger accumulation of propoxur in the fly compared to the honey bee. For example, the major metabolite recovered 1 hr after treatment was propoxur, amounting to 1.19 μg/g or 46% of the *internal extract*. Even after 12 hr the amount of propoxur present internally was 0.9 μg/g. While the propoxur which was formed in the house fly also was converted to secondary metabolites, the data indicate rapid formation of intoxicating quantities of propoxur which remained at high levels for at least 12 hr.

Metabolism in Honey Bees Treated With 300 μg/g 57 - The distribution and identification of metabolites recovered from honey bees 1 and 4 hr after treatment with 300 μg/g 57 are indicated in Table 8. As in the case of metabolism at the lower dosage, the parent compound 57 was by far the major radioactive constituent recovered in the *internal extract* of the honey bee, amounting to 5.56 and 6.73 μg/g and 1 and 4 hr following treatment, respectively. Thus at the two respective time intervals, 57 represented 70.4% and 51% of the total radioactivity present in the *internal extract*. The internal propoxur recoveries were 0.90 μg/g and 0.95 μg/g at 1 and 4 hr following treatment, respectively. Although the level of propoxur present internally was about 4-fold higher than that after treatment at the lower dosage, the amount of propoxur remained virtually constant for the two time periods, again suggesting the existence of a steady state situation for propoxur. It should be pointed out that the 4-fold difference in propoxur found at the higher dosage compared to the lower dosage is smaller than the 12-fold difference in dosages (300 vs 24.5 μg/g). Reduction in penetration at the higher dosage therefore appears to be one of the limiting factors which determines the amount of propoxur formed in the honey bee.

Metabolism in Honey Bees Treated With 4.5 μg/g propoxur - The metabolism of *N*-methyl-labeled [^{14}C]propoxur in honey bees was carried out to estimate the internal level of propoxur necessary for intoxication of the bee and to compare this level with that found in bees after treatment with 57 at 300 μg/g. Data for the distribution of propoxur metabolites in honey bees treated at the LD$_{50}$ dosage of 4.5 μg/g are given in Table 9. The major radioactive

TABLE 7. DISTRIBUTION AND IDENTIFICATION OF METABOLIC PRODUCTS RECOVERED FROM THE HOUSE FLY (S_{NAIDM}) AFTER TOPICAL APPLICATION OF 24.5 μg/g OF SULFENYL-PROPOXUR AT VARIOUS TIME INTERVALS

	1 hr		12 hr	
	%	μg/g	%	μg/g
Cage Rinse				
sulfenyl-propoxur	2.7	0.67	0.6	0.15
propoxur	0.2	0.03	0.1	0.02
N-CH_2OH-propoxur	0.1	0.02	-	-
O-de-*i*-propyl-propoxur	0.2	0.02	0.1	0.01
5-hydroxy-propoxur	0.3	0.04	-	-
others	0.1	-	0.4	-
unextractables	0.5	-	0.7	-
Total	4.1	-	1.9	-
Body Rinse				
sulfenyl-propoxur	70.7	17.31	36.8	9.02
propoxur	1.0	0.12	0.8	0.11
N-CH_2OH-propoxur	-	-	0.3	0.04
5-hydroxy-propoxur	-	-	0.8	0.12
others	-	-	9.2	-
Total	71.7	-	47.9	-
Internal Extracts				
Organosoluble extracts				
sulfenyl-propoxur	0.8	0.21	1.9	0.47
propoxur	8.1	1.06	6.2	0.81
N-CH_2OH-propoxur	0.2	0.02	0.4	0.06
O-de-*i*-propyl-propoxur	-	-	-	-
5-hydroxy-propoxur	-	-	0.9	0.13
others	0.3	-	0.8	-
Conjugates				
sulfenyl-propoxur	0.4	0.10	0.1	0.03
propoxur	1.0	0.13	0.7	0.09
N-CH_2OH-propoxur	0.6	0.09	1.1	0.15
O-de-*i*-propyl-propoxur	2.0	0.21	5.5	0.57
5-hydroxy-propoxur	1.9	0.27	3.5	0.49
others	0.9	-	10.3	-
unextractables	3.7	-	15.4	-
Total	19.9	-	46.8	-
Residue	1.3		1.2	
Total radioactivity recovered	97.0		97.8	

TABLE 8. DISTRIBUTION AND IDENTIFICATION OF METABOLIC PRODUCTS RECOVERED FROM HONEY BEES AFTER TOPICAL APPLICATION OF 300 µg/g OF SULFENYL-PROPOXUR AT VARIOUS TIME INTERVALS

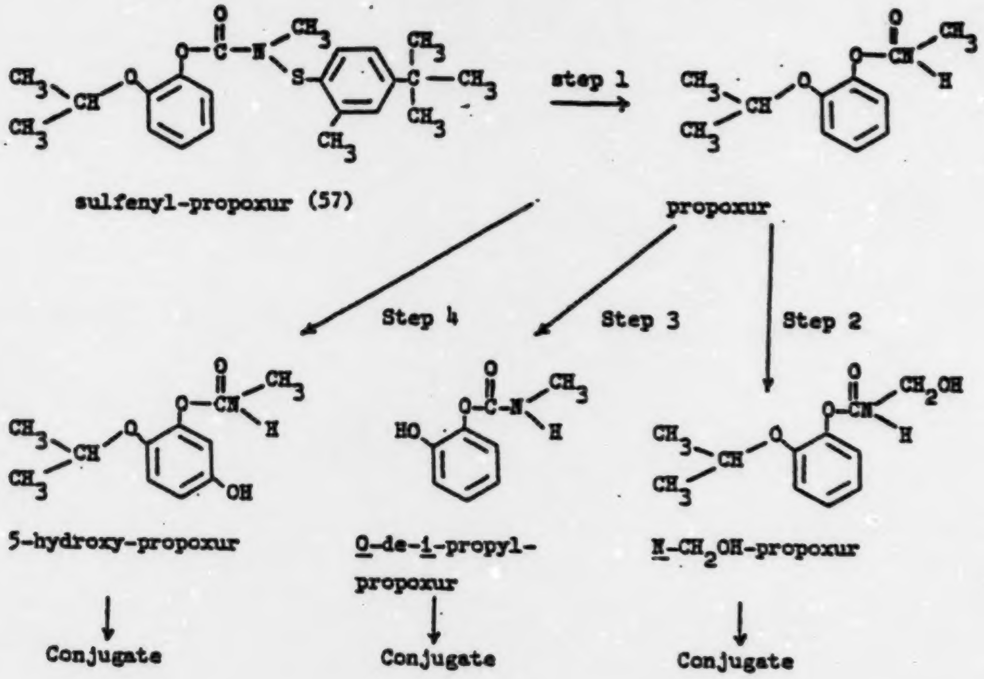
	1 hr		4 hr	
	%	µg/g	%	µg/g
Cage Rinse				
sulfenyl-propoxur	10.0	30.13	21.7	65.18
propoxur	0.2	0.40	0.4	0.60
N-CH$_2$OH-propoxur	-	-	-	-
O-de-*i*-propyl-propoxur	-	-	-	-
others	0.1	-	0.1	-
unextractables	1.1	-	0.5	-
Total	11.4		22.7	
Body Rinse				
sulfenyl-propoxur	77.1	231.42	69.0	207.06
propoxur	0.6	0.99	0.5	0.77
Total	77.7	-	69.5	-
Internal Extracts				
Organosoluble extracts				
sulfenyl-propoxur	1.8	5.39	2.1	6.31
propoxur	0.6	0.90	0.6	0.95
N-CH$_2$OH-propoxur	0.1	0.12	0.6	1.10
O-de-*i*-propyl-propoxur	-	-	0.4	0.50
5-hydroxy-propoxur	-	-	-	-
others	-	-	-	-
Conjugates				
sulfenyl-propoxur	0.1	0.17	0.1	0.42
propoxur	-	-	-	-
N-CH$_2$OH-propoxur	-	-		
O-de-*i*-propyl-propoxur	-	-	0.1	0.09
5-hydroxy-propoxur	-	-	-	-
others	-	-	-	-
unextractables	0.1	-	0.4	-
Total	2.7	-	4.3	-
Residue	1.9	-	1.2	-
Total radioactivity recovered	93.7	-	97.7	-

TABLE 9. DISTRIBUTION AND IDENTIFICATION OF METABOLIC PRODUCTS RECOVERED FROM THE HONEY BEE AFTER TOPICAL APPLICATION OF 4.5 µg/g PROPOXUR AT VARIOUS TIME INTERVALS.

	1 hr		4 hr	
	%	µg/g	%	µg/g
Cage Rinse				
propoxur	10.2	0.46	12.1	0.54
N-CH$_2$OH-propoxur	-	-	0.1	0.01
O-de-*i*-propyl-propoxur	-	-	0.1	0.01
5-hydroxy-propoxur	-	-	-	-
others	0.8	-	0.1	-
unextractables	-	-	0.7	-
Total	11.0	-	13.1	-
Body Rinse				
propoxur	51.0	2.29	23.4	1.05
others	0.7	-	0.3	-
Total	51.7	-	23.7	-
Internal Extracts				
Organosoluble extracts				
propoxur	22.0	0.99	15.5	0.70
N-CH$_2$OH-propoxur	3.7	0.18	14.4	0.70
O-de-*i*-propyl-propoxur	1.6	0.06	6.8	0.25
5-hydroxy-propoxur	0.2	0.01	0.5	0.02
others	0.3	-	1.3	-
Conjugates				
propoxur	2.0	0.09	3.6	0.16
N-CH$_2$OH-propoxur	1.8	0.09	5.7	0.26
O-de-*i*-propyl-propoxur	0.8	0.03	2.1	0.08
5-hydroxy-propoxur	0.1	0.01	0.1	0.01
others	0.3	-	0.6	-
unextractables	3.1	-	8.3	-
Total	35.9	-	58.9	-
Residue	0.8	-	1.5	-
Total radioactivity recovered	99.4	-	97.2	-

constituent present internally in the bee was propoxur, amounting to 67% and 32% of the total internal radioactivity 1 and 4 hr after treatment, respectively. These percentages translate to 1.08 μg/g and 0.86 μg/g propoxur at the two respective intervals. Therefore, in terms of actual quantities the levels of propoxur present internally in the honey bee treated with 4.5 μg/g propoxur were about equal to the levels of propoxur present after treatment with 300 μg/g 57.

The metabolic pathway for 57 in the honey bee and house fly may be summarized by the following scheme.



Summary - While replacement of the carbamate hydrogen atom of a toxic methylcarbamate insecticide by a sulfenyl moiety generally results in products which are substantially safer to mammals than the parent methylcarbamate, most of the sulfenylated derivatives which were examined for toxicity to the honey bee were highly toxic to this insect. Of the 34 different sulfenylated derivatives of propoxur, carbofuran, and carbaryl evaluated only three showed a toxicity ratio greater than one although many were safer to bees than the parent methylcarbamate. The most notable exception was the 2-methyl-4-*t*-butylphenylsulfenyl derivative of propoxur (57) which was more than 178-fold safer to the honey bee than propoxur. Although the 4-*t*-butyl moiety in the ring appeared to introduce the greatest amount of honey bee safety in these derivatives, no obvious relationship between the structure of the carbamate derivative and honey bee toxicity was apparent.

While the pathways in the honey bee and house fly are qualitatively similar, there are enough quantitative differences to account for the difference in the toxicity of 57 to the two insects. It is apparent that penetration of 57 and its subsequent conversion into propoxur are rate limiting steps in the metabolism of 57 in the honey bee. Because of the rapid conversion of propoxur into secondary metabolites (steps 2, 3 and 4 in the scheme) the amount of propoxur accumulating in the bee was restricted to a low, steady-state level and, therefore, intoxication did not occur. In house flies, however, penetration and conversion of 57 into propoxur was fast and intoxicating levels of propoxur accumulated within a short period of time after treatment.

4. Selective Toxicity of Phosphoramidothioate Esters.

Introduction - Although methamidophos has many virtues as an insecticide, unfortunately it is also somewhat toxic to warm-blooded animals (oral rat and mice LD_{50} are 20 and 14 mg/kg, respectively[10]. In contrast, the *N*-acetyl (acephate) and *N*-propionyl derivatives of methamidophos, while still retaining the good insecticidal and acaricidal properties of methamidophos, are of low mammalian toxicity (rat oral LD_{50} of 900 and >1000 mg/kg, respectively). Selectivity, however, is reversed when the acyl chain length is increased, and the hexanoyl derivative, with a mouse oral LD_{50} of 35 mg/kg, is slightly more toxic to the mouse than it is to house flies.

Owing to the interesting contrast in insecticidal activity and mammalian toxicity between the hexanoyl derivative and the smaller chain acyl derivatives, a study of the comparative metabolism of these compounds in a mammal and insect was undertaken to establish the basis for their toxicological properties.

Toxicological Properties - Toxicological data for *O*,*S*-dimethyl propionyl- (I) and hexanoylphosphoramidothioate (II) are presented in Table 8. Results for mouse and house fly toxicity are in general agreement with those reported by Magee[10] for the rat and house fly.

Anticholinesterase data in Table 1 show that both I and II are very poor inhibitors of house fly-head (HFAChE) and bovine erythrocyte acetylcholinesterase (BAChE). For all practical purposes I and II may be considered inert to these enzymes. Methamidophos (III) is substantially more effective as an anticholinesterase than I or II, but is relatively poor compared to other organophosphorus esters such as paraoxon and tetraethyl pyrophosphate.

TABLE 10. TOXICOLOGICAL PROPERTIES OF O,S-DIMETHYL PROPIONYL- (I), HEXANOYLPHOSPHORAMIDOTHIOATE (II), AND METHAMIDOPHOS (III).

	I	II	III (Methamidophos)
Mouse oral LD_{50} (mg/kg)[a]	350	35	14.0
Mouse i.p. LD_{50} (mg/kg)	92[b]	25[c]	5.3
House fly LD_{50}, S_{NAIDM} LD_{50} ($\mu g/g$)	1.1	38	1.20
AChE inhibition, bovine erythrocyte, k_i (M^{-1} min^{-1})	<1	<1	1.8×10^3
AChE inhibition, house fly-head, k_i (M^{-1} min^{-1})	1.3	2.4	2.5×10^3

[a] Corn oil was used as the carrier.

[b] Compound was dissolved in saline (0.95% sodium chloride).

[c] Compound was dissolved in propylene glycol–saline (1:1, v/v).

Metabolism in the House Fly - Results for the metabolism of I in S_{NAIDM} flies are presented in Table 11 and those for II in Table 12. The parent compound and only two metabolites were isolated from flies treated with I. These were methamidophos (III, O,S-dimethyl phosphoramidothioate) and the O-demethylated derivative of I (IV, hydrogen S-methyl propionylphosphoramidothioate). Of the three compounds found in the internal extract, methamidophos (III) was by far the most prominent, representing approximately 23% of the recovered or 22% of the applied radioactivity. Owing to the substantially higher anticholinesterase activity of III compared to I, it is probable that III is the agent responsible for intoxication.

Compared to I, the metabolism of II in the house fly is more complex and at least five metabolites, in addition to the parent compound, were isolated. The metabolites were methamidophos (III), the O-demethylated derivative of II (V, hydrogen S-methyl hexanoylphosphoramidothioate), O-demethylated methamidophos (VI, hydrogen S-methyl phosphoramidothioate), O,S-dimethyl phosphorothioic acid (VII), and an unknown (VIII) of similar but slightly different chromatographic properties as methamidophos. All six compounds, including the parent material II, were detected in the internal extract and, as in the case of I, methamidophos was most prominent although on a percentage basis less was found than with I (4.55% of recovered radioactivity compared to 23.3% for I). However, owing to the 39-fold larger dosage of II compared to I, 5.7-fold more methamidophos was detected in the internal fraction of flies treated with II (after correction for molecular weights).

TABLE 11. METABOLIC PRODUCTS RECOVERED FROM HOUSE FLIES 6 HR AFTER TOPICAL APPLICATION OF 0.535 μg/g S-[^{14}C]METHYL O-METHYL PROPIONYLPHOSPHORAMIDOTHIOATE (I)

Metabolite	Recovery		
	Equivalents of I (μg/g)	Recovered radioactivity (%)	Applied radioactivity (%)
Cage rinse			
I Parent compound	0.023	4.6	4.3
IV *O*-Demethyl-I	0.004	0.8	0.7
Body rinse			
I Parent compound	0.170	34.1	31.7
III Methamidophos	0.018	3.7	3.4
IV *O*-Demethyl-I	0.011	2.2	2.0
Internal extract			
I Parent compound	0.064	12.9	12.0
III Methamidophos	0.116	23.3	21.6
IV *O*-Demethyl-I	0.005	1.0	0.9
Unextractable	0.050	10.1	9.4
CO_2	0.036	7.3	6.8
Total	0.497	100	92.8

TABLE 12. METABOLIC PRODUCTS RECOVERED FROM HOUSE FLIES 6 HR AFTER TOPICAL APPLICATION OF 20.78 μg/g S-[^{14}C]METHYL O-METHYL HEXANOYLPHOSPHORAMIDOTHIOATE (II)

Metabolite	Recovery		
	Equivalents of II (μg/g)	Recovered radioactivity (%)	Applied radioactivity (%)
Cage rinse			
II Parent compound	0.99	5.7	4.8
III Methamidophos	0.07	0.4	0.3
V *O*-Demethyl-II	0.15	0.9	0.8
VI $(CH_3O)(CH_3S)P(O)OH$	0.02	0.1	0.1
VII $(HO)(CH_3S)P(O)NH_2$	0.04	0.2	0.2
VIII Unknown	0.06	0.4	0.3
Conjugates	0.01	<0.1	<0.1
Body rinse			
II Parent compound	8.65	49.6	41.7
III Methamidophos	0.15	0.9	0.8
V *O*-Demethyl-II	0.61	3.5	3.0
VI $(CH_3O)(CH_3S)P(O)OH$	0.16	0.9	0.8
VII $(HO)(CH_3S)P(O)NH_2$	0.14	0.8	0.7
VIII Unknown	0.16	0.9	0.8
Conjugates	0.03	0.1	0.1
Internal extract			
II Parent compound	2.54	14.5	12.2
III Methamidophos	0.79	4.6	3.8
V *O*-Demethyl-II	0.34	1.9	1.6
VI $(CH_3O)(CH_3S)P(O)OH$	0.07	0.4	0.3
VII $(HO)(CH_3S)P(O)NH_2$	0.04	0.2	0.2
VIII Unknown	0.39	2.3	1.9
Conjugates	0.03	0.1	0.1
Unextractable	0.36	2.1	1.7
CO_2	1.65	9.4	7.9
Total	17.45	100.0	84.2

Metabolism in the White Mouse - Results for the metabolism of I in a single white mouse 6 hr after oral treatment at a dosage of 50.16 mg/kg are presented in Table 13. At this time, about two-thirds of the administered radioactivity was eliminated from the mouse as urinary, fecal, and respiratory (presumably as carbon dioxide) products. Another 18.7% of applied radioactivity was present in mouse tissue. Total recovery of radioactivity was 85.4%.

TABLE 13. METABOLIC PRODUCTS RECOVERED FROM THE WHITE MOUSE 6 HR AFTER ORAL ADMINISTRATION OF 50.16 mg/kg S-[^{14}C]METHYL O-METHYL PROPIONYLPHOSPHORAMIDOTHIOATE (I)

Metabolite	Recovery		
	Equivalents of I (μg/g)	Recovered radioactivity (%)	Applied radioactivity (%)
Urine			
I Parent compound	13.05	30.5	26.0
III Methamidophos	0.94	2.2	1.9
IV *O*-Demethyl-I	1.24	2.9	2.5
Feces			
I Parent compound	6.10	14.2	12.1
III Methamidophos	0.35	0.8	0.7
IV *O*-Demethyl-I	0.43	1.0	0.9
CO_2	11.31	26.5	22.6
Remaining in mouse tissue	9.36	21.9	18.7
Total	42.78	100	85.4

As in the case of house flies, I and only two metabolites were recovered from the mouse after treatment with I. These were present as both urinary and fecal products and were methamidophos and IV. Of the radioactivity isolated, I was present in greatest abundance with a combined total of more than 38% of the applied dosage present in urine and feces. Methamidophos was the least abundant metabolite, totaling about 2.5% in urine and feces. Thus, it appears that I is quite stable to metabolism and over a third of the applied dosage passed through the mouse unchanged. This may be attributable to the high water solubility of I. The most significant pathway for the metabolism of I appears to be through a process leading to the generation of carbon dioxide (approximately 23% of the applied dosage).

Data for the metabolism of II in white mice treated at dosages of 18.50 and 14.88 mg/kg are presented in Table 14. Compared to I, elimination of radioactivity after treatment with II was slow, and substantial amounts of radioactivity remained in the mouse stomach 6 hr after treatment with 18.50 mg/kg of II. Because of this, another mouse was dosed (14.9 mg/kg) and analysis of metabolic products was conducted 24 hr after treatment. At 6 hr, only 20% of the applied radioactivity was eliminated via the urine and, surprisingly, no radioactivity was present in the feces. However, 31% of

TABLE 14. METABOLIC PRODUCTS RECOVERED FROM THE WHITE MOUSE 6 AND 24 HR AFTER ORAL ADMINISTRATION OF 18.50 AND 14.88 MG/KG, RESPECTIVELY, OF S-[^{14}C]METHYL O-METHYL HEXANOYLPHOSPHORAMIDOTHIOATE (II).

Metabolite		Recovery		
		Equivalents of II (μg/g)	Recovered radioactivity (%)	Applied radioactivity (%)
	6 hr after treatment at 18.5 mg/kg			
Urine				
II	Parent Compound	0.19	1.2	1.0
III	Methamidophos	2.53	15.8	13.7
V	*O*-Demethyl-II	0.19	1.2	1.0
VI	$(CH_3O)(CH_3S)P(O)OH$	0.09	0.5	0.5
VII	$(HO)(CH_3S)P(O)NH_2$	0.20	1.3	1.1
VIII	Unknown	0.61	3.8	3.3
Stomach				
II	Parent compound	4.99	31.1	27.0
III	Methamidophos	0.31	1.9	1.7
V	*O*-Demethyl-II	0.44	2.8	2.4
	CO_2	4.56	28.5	24.7
Remaining in mouse tissue		1.91	11.9	10.3
Total		16.02	100	86.7
	24 hr after treatment at 14.88 mg/kg			
Urine				
II	Parent compound	0.07	0.5	0.5
III	Methamidophos	2.72	21.1	18.3
V	*O*-Demethyl-II	0.19	1.5	1.3
VII	$(HO)(CH_3S)P(O)NH_2$	1.03	8.0	6.9
VIII	Unknown	0.76	5.9	5.1
	Conjugate	0.01	0.1	0.1
Feces				
II	Parent compound	0.05	0.4	0.3
III	Methamidophos	1.11	8.6	7.4
V	*O*-Demethyl-II	0.10	0.8	0.7
VII	$(HO)(CH_3S)P(O)NH_2$	0.03	0.3	0.2
VIII	Unknown	0.65	5.0	4.4
	Conjugate	0.31	2.4	2.1
	CO_2	5.85	45.4	39.3
Total		12.88	100	86.6

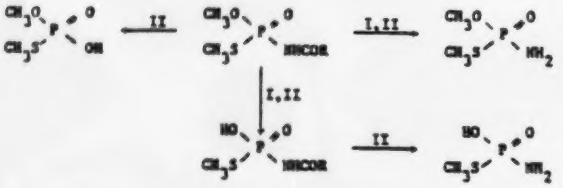
the applied radioactivity still remained in the stomach and almost 25% was expired as carbon dioxide. At 24 hr following treatment with 14.88 mg/kg II, about 87% of the applied radioactivity was eliminated in the excreta or as carbon dioxide.

At the 6-hr period the metabolism of II in the mouse was qualitatively similar to that in house flies. The same five metabolites, methamidophos, V, VI, VII, and the unknown VIII, were isolated as urinary products. In contrast to I, where methamidophos was a minor metabolite, methamidophos was the major metabolite present in urine after treatment with II and amounted to 13.7% of the applied dosage. In terms of actual quantity, the amount of methamidophos isolated from the mouse (urine and stomach) treated with II was 1.68 mg/kg. This is substantially more than 0.92 mg/kg, the amount of methamidophos isolated from the mouse treated with 50.16 mg/kg of I. Thus, even though the dosage of II on a weight basis was 2.7-fold less than I, almost 2-fold more methamidophos was recovered from the mouse treated with II.

The 24-hr data largely supported the 6-hr results, although there were minor differences. Metabolite VI (O,S-dimethyl phosphorothioic acid) was not detected but this was of minor significance even in the 6-hr analysis. Metabolism to carbon dioxide, accounting for 39% of the applied radioactivity, was by far the most important pathway of degradation. Substantially more methamidophos, amounting to 25.7% of the applied radioactivity, was detected in the 24-hr urine and feces. In actual amounts this corresponds to 2.28 mg/kg of methamidophos.

While I passed through the mouse rapidly and large amounts were recovered unchanged in the urine, this was not the case with II. This difference in behavior between I and II may be attributable to differences in their water solubility and lipid-water partitioning properties. The partition constants for I and II between octanol-water are 0.53 and 6.45, respectively.

Summary - Based on the data in Tables 11-14 it is apparent that the metabolism of O,S-dimethyl propionylphosphoramidothioate (I) in the white mouse and house fly is qualitatively and quantitatively different from that of the hexanoyl analog (II). Qualitative behavior of I and II in the mouse and house fly is summarized in the metabolic scheme below. According to the scheme,



methamidophos and the O-demethyl derivatives are obtained as metabolites from both I (R = C_2H_5) and II (R = C_5H_{11}) but O,S-dimethyl phosphorothioic acid and O-demethyl methamidophos are obtained only from II. Further, since I and II are both metabolized to methamidophos, it is assumed that O,S-dimethyl phosphorothioic acid (VI) and O-demethyl methamidophos (VII) are generated from II and O-demethyl-II, respectively, and not from methamidophos. This difference in the metabolic behavior between I and II is probably attributable to their contrasting lipophilic or hydrophilic properties, with I favoring water over octanol and II favoring octanol over water. This is evident in the large amounts of I (26% of applied dosage in the urine) which passed through the mouse unchanged, compared to substantially lesser amounts for II (1% of applied dosage). In contrast to most organophosphorus and other organic insecticides, I, owing to its high water solubility, may pass through the mouse unchanged.

The major difference of significance in the metabolism of I and II in the mouse appears to be in the larger amounts of methamidophos formed from II compared to I. Methamidophos is highly toxic to the mouse (LD_{50} 14 mg/kg), and probably is the agent responsible for intoxication when the mouse is treated with either I or II. The small amounts of methamidophos formed from I readily account for the safety of I to mice. On the same basis, the relatively high toxicity of II to mice may be attributed to the substantial quantity of methamidophos formed in the mouse treated with II.

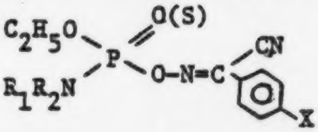
5. References.

1. Fahmy, M.A.H., Y.C. Chiu, T.R. Fukuto, J. Agr. Food Chem. 22, 59 (1974).
2. Black, A.L., Y.C. Chiu, M.A.H. Fahmy, T.R. Fukuto, J. Agr. Food Chem. 21, 747 (1973).
3. Fukuto, T.R., A.L. Black, Y.C. Chiu, M.A.H. Fahmy, Environ. Quality Safety, Suppl. Vol. III, 393 (1975).
4. Black, A.L., Y.C. Chiu, T.R. Fukuto, T.A. Miller, Pestic. Biochem. Physiol. 3, 435 (1973).
5. Krieger, R.I., P.W. Lee, M.A.H. Fahmy, M. Chen, T.R. Fukuto, Pestic. Biochem. Physiol. 6, 1 (1976).
6. Chiu, Y.C., A.L. Black, T.R. Fukuto, Pestic. Biochem. Physiol. 5, 359 (1975).
7. Metcalf, R.L., M. Frederickson, J. Econ. Entomol. 58 143 (1965).
8. Camp, H.B., T.R. Fukuto, R.L. Metcalf, J. Agr. Food Chem. 17, 243 (1969).
9. Chiu, Y.C., A.L. Black, T.R. Fukuto, Pestic. Biochem. Physiol. 5, 359 (1975).
10. Magee, P.S., Residue Rev. 53, 3 (1974).

C. STRUCTURE-ACTIVITY RELATIONSHIPS

1. Hydrolytic and Toxicological Properties of Ethyl α-Cyanobenzaldoxime Phosphoramidates.

Introduction - Because of the desired order of selectivity demonstrated by phoxim (*O*,*O*-diethyl *O*-α-cyanobenzaldoxime phosphorothioate) and analogs and the limited amount of information concerning the relationship of chemical structure and biological activity of phosphoramidate esters, it was of interest to examine a series of phosphoramidates containing the α-cyanobenzaldoxime leaving group which is present in phoxim. This section is concerned with the synthesis and toxicological evaluation of phosphoramidates of the general structure shown below where R_1 and R_2 are hydrogen or small alkyl groups and X is H, Cl, NO_2, Me, or MeO groups.



Hydrolysis - Second-order hydrolysis constants (k_b) for the various benzaldoxime phosphoramidates are presented in Table 1.

For the phosphoramidates without ring substituents, i.e., compounds *1*-*7*, rate constants were determined for the first 5 compounds. The *t*-butyl- (*6*) and dimethylamido (*7*) analogs were very stable and no evidence of hydrolysis was obtained for either compound in 0.05 M sodium hydroxide after 24 hr. Regression analysis of the data with Taft's[1] σ^* and E_s constants for compounds *1*-*5* resulted in very good correlation with each parameter according to equations 1 and 2.

$$\log k_b = 9.10\,\sigma^* - 1.48 \qquad (1)$$

$$\underline{r} = 0.989 \quad \underline{n} = 5 \quad \underline{F} = 138.8$$

$$\log k_b = 3.61\,E_s - 1.60 \qquad (2)$$

$$\underline{r} = 0.981 \quad \underline{n} = 5 \quad \underline{F} = 78.0$$

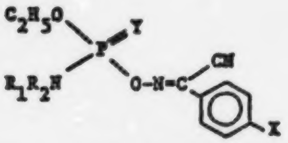
n is the number of compounds, *r* is the correlation coefficient, and the *F* value is the statistical measure of the significance of the correlation.

Unfortunately, there was a high degree of colinearity between σ^* and E_s for the amido substituents in compounds *1*-*5* (*r* = 0.99) and it was difficult to establish which of the two parameters was more important in correlating the hydrolysis data. Inclusion of both σ^* and E_s in the regression analysis gave equation 3.

$$\log k_b = 8.84\,\sigma^* + 0.10\,E_s - 1.49 \qquad (3)$$

$$\underline{r} = 0.989 \quad \underline{n} = 5 \quad \underline{F} = 46.3$$

TABLE 1. TOXICOLOGICAL AND HYDROLYTIC PROPERTIES OF SUBSTITUTED ETHYL α-CYANOBENZALDOXIME PHOSPHORAMIDATES



	Compound				k_i ($M^{-1}min^{-1}$)		LD_{50} (mg/kg)		$k_b \times 10^{-2}$	
No.	R_1	R_2	X	Y	HFAChE	BAChE	House fly	Mouse	($M^{-1}min^{-1}$)	log P
1	H	H	H	O	2.0×10^8	1.7×10^8	>500	-	3.8×10^5	0.72
2	H	Me	H	O	2.8×10^7	4.5×10^6	11.7	10	2.15	0.96
3	H	Et	H	O	9.5×10^6	9.6×10^5	17.5	75	1.41	1.46
4	H	_n_-Pr	H	O	7.5×10^6	5.0×10^5	22.5	>500	1.35	1.96
5	H	_i_-Pr	H	O	2.1×10^5	1.6×10^5	45	150-200	0.261	1.76
6	H	_t_-Bu	H	O	8.8×10^4	2.0×10^4	>500	-	0[a]	2.14
7	Me	Me	H	O	1.3×10^5	1.2×10^4	90	<50	0[a]	3.29
8	H	Me	Cl	O	1.3×10^7	1.2×10^6	3.9	50-75	0.989	2.50
9	H	Et	Cl	O	3.1×10^6	3.5×10^5	3.6	>500	0.605	3.00
10	H	_n_-Pr	Cl	O	8.4×10^5	1.3×10^5	8.3	>500	0.636	3.50
11	H	Et	NO_2	O	7.1×10^6	8.2×10^5	>500 (7.0)[b]	-	1.73	1.76
12	H	Et	MeO	O	2.6×10^6	8.4×10^4	>500 (7.3)[b]	-	-	2.39
13	H	Et	Me	O	3.0×10^6	1.7×10^5	>500 (11.5)[b]	-	0.818	1.46
14	H	Me	H	S	-	-	>300	-	-	-

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[a] No detectable evidence for hydrolysis after 24 hr in 0.05 _M_ NaOH.
[b] With piperonyl butoxide 5:1 (w/w).

The coefficients in the equation indicate a greater dependence on σ^* than E_s but steric effects undoubtedly become more important as the size of the amido substituent becomes larger. This is illustrated by data obtained with 6 (NH-t-Bu) which showed no evidence of hydrolysis after 24 hr in 0.05 M sodium hydroxide. From equation 1, using $\sigma^* = 0.19$ for t-Bu, the calculated k_b for 6 was 1.78 $M^{-1}min^{-1}$. Based on this rate constant, the half-life ($t_{1/2}$) of 6 in 0.05 M sodium hydroxide was calculated to be 7.8 min, a value which is at least 2 orders of magnitude greater than the actual half-life.

Although only a few ring substituted benzaldoxime phosphoramidates were prepared and examined for hydrolysis (8-13), analysis of the data showed no trend between structure and hydrolysis rates. This is evident from the k_b values for 3, 9, 11, and 13, i.e., analogs which are identical except for the ring substituent. Owing to overlaps in relevant absorption peaks, k_b for 12 (p-OCH_3) could not be determined. Since alkaline hydrolysis occurs by cleavage of the phosphorus-oxime oxygen bond, ring substituents which are electron-withdrawing should increase hydrolysis rates. While the p-nitro derivative (11), with $k_b = 173.9\ M^{-1}min^{-1}$, was most susceptible to hydrolysis, the p-chloro analog (9) was least susceptible. In fact, 9 with the electron-withdrawing chlorine substituent, was distinctly more stable to alkaline hydrolysis than the unsubstituted phosphoramidate (3) and compound 13, containing the electron-donating p-methyl group.

Cholinesterase Inhibition - Bimolecular constants (k_i) for the inhibition of acetylcholinesterase show that the various phosphoramidates were potent anticholinesterases and, in general, were stronger inhibitors of HFAChE than BAChE. Evidence for greater anticholinesterase activity toward HFAChE is shown graphically in Fig. 1 which gives the relation between log k_i and -log k_b for the N-alkylphosphoramidates for which k_b and k_i values were obtained. Overall, the plot reveals a linear relationship between the logarithms of the rate constants for inhibition and hydrolysis, and anticholinesterase activity against HFAChE is about 10-fold greater than against BAChE. Progressive increase in the size of the amido alkyl substituent resulted in a decrease in anticholinesterase activity (compare compounds 1-6 and 8-10) against both enzymes.

Regression analysis of the inhibition data for compounds 1-7 against BAChE with σ^*, E_s and π resulted in equation 4 which gave best fit to the data.

$$\log k_i = 2.34\,E_s + 0.8\,\sigma^* + 4.10 \qquad (4)$$

$$r = 0.990 \quad n = 7 \quad F = 98.2$$

In spite of their colinearity, the inclusion of σ^* and E_s together in the equation was statistically significant at the 0.05 level of probability, providing evidence that anticholinesterase activity was governed mainly by polar and steric effects associated with the amido moiety.

Analysis of the data for inhibition of HFAChE (1-7) resulted in equation 5 which gave best fit to the data.

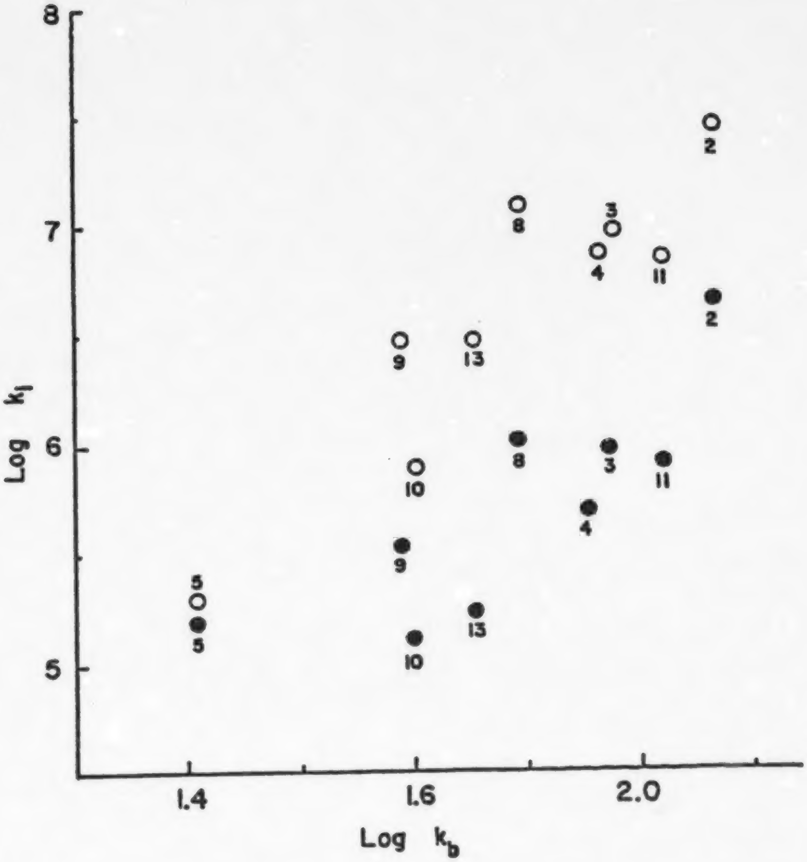


FIG. 1. Relation between log k_b for alkaline hydrolysis and log k_i for the inhibition of HFAChE(O) and BAChE(●).

$$\log k_i = 1.33\ E_s + 5.23 \qquad (5)$$

$$\underline{r} = 0.923 \quad \underline{n} = 7 \quad \underline{F} = 28.8$$

Inclusion of σ^* in the regression analysis surprisingly had no effect on the correlation, indicating the absence of polar influence by the amido moieties on HFAChE inhibition.

Analysis of the inhibition data by the ring substituted phosphoramidates (**3**, **9**, **11**, **12**, **13**) against HFAChE and BAChE resulted in poor correlation ($\underline{r}$ = 0.873 and 0.814, respectively) with σ and π. The results were not statistically significant at the 0.05 level of probability.

Toxicity - The phosphoramidates in Table 1 showed variable but interesting toxicity to house flies and mice. For the compounds without substituents in the phenyl ring (**1**-**7**), increase in size of the amido alkyl substituent resulted in a gradual decrease in house fly toxicity. Despite its high anticholinesterase activity the unsubstituted amido analog 1 was nontoxic to flies and this was probably attributable to its high hydrolytic instability. Compared to flies, a drastic decrease in mouse toxicity with increasing alkyl size was observed (compare **2**, **3**, and **4**).

An almost perfect linear relationship was obtained in correlating house fly toxicity with π and k_i values for HFAChE (equation 6).

$$\log LD_{50} = 0.16\ \pi - 0.21 \log k_i + 2.56 \qquad (6)$$

$$\underline{r} = 0.999 \quad \underline{n} = 5 \quad \underline{F} = 7555$$

Compounds included in equation 6 are those for which finite house fly LD_{50} values were obtained, i.e., **2**, **3**, **4**, **5**, and **7**. Although the high degree of correlation may be purely fortuitous owing to the small number of compounds, nevertheless, the equation points out the equal importance of hydrophobic effects and anticholinesterase activity in determining toxicity.

Placement of substituents on the phenyl ring had a profound effect on house fly toxicity. For example, introduction of a chlorine substituent in the *para* position increased house fly toxicity 3- to 5-fold (compare **2**, **3**, **4** with **8**, **9**, **10**) but substitution with nitro, methoxy, and methyl groups resulted in compounds which were nontoxic at 500 µg/g. The latter three compounds (**11**, **12**, **13**), however, were strongly synergized by cotreatment with 5:1 parts of piperonyl butoxide. In fact, the synergism of more than 70-fold observed for **11** and **12** with piperonyl butoxide is unusual for an organophosphorus ester and is even greater than the level of synergism observed for carbamate esters. Because of their high anticholinesterase activity the low toxicity of **11**, **12**, and **13** when applied alone to house flies was surprising. These compounds evidently are highly susceptible to oxidative detoxication, as indicated by their strong response to piperonyl butoxide.

For reasons which are not obvious, the p-chloro substituted phosphoramidates were less toxic to mice than the corresponding unsubstituted analogues (compare 2, 3, 4 with 8, 9, 10).

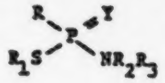
Summary - A series of ethyl alkylphosphoramidate esters of α-cyanobenzaldoxime and ring-substituted α-cyanobenzaldoximes were synthesized and examined for alkaline hydrolysis, anticholinesterase activity, and toxicity to house flies and mice. The compounds showed variable toxicity to house flies and mice and were effective inhibitors of acetylcholinesterase. Satisfactory correlation was obtained between alkaline hydrolysis rate constants and anticholinesterase activity with Taft's σ^* and E_s values. Toxicity to house flies was related to anticholinesterase activity and Hansch's π constants. Several of the compounds were of low toxicity to the white mouse but were highly effective against the house fly. The interesting toxicological properties of the α-cyanobenzaldoxime phosphoramidates justify further examination of compounds of this type as potential insecticides.

2. Insecticidal Properties of Phosphonamidothioate Esters and Derivatives.

Introduction - In an earlier study[2], we described the outstanding insecticidal activity of a few phosphonamidothioate esters related to methamidophos. For example, S-ethyl ethylphosphonamidothioate with a house fly LD_{50} of 0.64 μg/g was about 2-fold more toxic to the house fly than methamidophos. Unfortunately, this compound was also about 2-fold more toxic to the white mouse. Because of the high degree of selectivity achieved by converting methamidophos to acephate, i.e., by acetylation, it was of interest to examine the effect of acylation on the mammalian toxicity and insecticidal activity of S-alkyl alkylphosphonamidothioate esters. This section is concerned with the synthesis and evaluation of the toxicological properties of a series of acylated S-alkyl alkylphosphonamidothioates and some related esters.

Toxicological Properties - Data for the toxicity of the various S-alkyl alkylphosphonamidothioates and derivatives are presented in Table 2. The data show that highest toxicity to house flies is associated with the S-methyl or S-ethyl P-alkylphosphonamidothioates containing small P-alkyl moieties (15, 18, 19, and 20). Increasing the chain length of the P-alkyl moiety resulted in abrupt reduction in house fly toxicity with the P-butyl analog (24). Branching in the P-alkyl moiety drastically reduced house fly toxicity and the P-isopropyl analog 23 was devoid of insecticidal activity. As in the case of the S-alkyl O-alkyl phosphoramidothioates, substitution on the nitrogen atom greatly reduced insecticidal activity of the S-methyl P-methylphosphonamidothioates (compare 15, 16, and 17) and the N,N-dimethyl analog of the S-ethyl P-ethylphosphonamidothioate (21) with a house fly LD_{50} of 60 μg/g was moderately active. It was, however, about 100-fold less effective than the unsubstituted compound 20.

TABLE 2. TOXICOLOGICAL PROPERTIES OF PHOSPHONAMIDOTHIOATES AND DERIVATIVES



No.	R	R_1	R_2	R_3	Y	House fly LD_{50} (μg/g) alone	House fly LD_{50} (μg/g) + PB[a]	Mouse LD_{50} (mg/kg)
15	Me	Me	H	H	O	1.0	0.63	10
16	Me	Me	H	Me	O	205	120	-
17	Me	Me	Me	Me	O	>500	-	>100
18	Me	Et	H	H	O	0.65	-	-
19	Et	Me	H	H	O	0.81[b]	0.47	16
20	Et	Et	H	H	O	0.64[b]	-	16[b]
21	Et	Et	Me	Me	O	60[b]	-	-
22	n-Pr	Me	H	H	O	4.5	3.0	30
23	i-Pr	Me	H	H	O	>500	-	>100
24	n-Bu	Me	H	H	O	52	-	35
25	Et	Me	H	$C(O)CH_3$	O	52	19.7	>100
26	Et	Et	H	$C(O)CH_3$	O	153	33	>100
27	Et	Me	H	$C(O)C_2H_5$	O	4.5	-	>100
28	Et	Et	H	$C(O)C_2H_5$	O	150	3.8	>100
29	Et	Et	H	$C(O)C_3H_7$-n	O	92	15.5	>100
30	Et	Me	H	$C(O)C_3H_7$-i	O	114	73	>100
31	Et	Et	H	$C(O)C_3H_7$-i	O	>500	99	-
32	Et	Me	H	$C(O)C_4H_9$-n	O	87	18	>100
33	Et	Et	H	$C(O)C_5H_{11}$-n	O	350	52	-
34	Et	Me	H	$C(O)CH=CMe_2$	O	>500	>500	>100
35	Me	Me	H	$\overbrace{CHO(CH_2)_2CH_2}$	O	45	11	<10
36	Me	Me	H	H	S	15.8	8.5	-
37	Et	Me	H	H	S	11.5	-	55
38	Et	Me	H	Me	S	250	-	>100
39	Et	Me	Me	Me	S	>500	-	>100

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[a] Piperonyl butoxide was applied at a constant dose of 500 μg/g.

[b] From Quistad et al.[2]

The outstanding house fly toxicity of the simple S-alkyl alkylphosphonamidothioates prompted us to examine the toxicological properties of their acylated derivatives.

In general, acylation of the S-methyl or S-ethyl methyl- and ethylphosphonamidothioates resulted in compounds (25-34) which were relatively ineffective against house flies compared to the parent phosphonamidothioates. Whereas acylation of methamidophos with the acetyl or propionyl moieties gave products, e.g., acephate, which were equal to methamidophos in insecticidal activity, acylation of the phosphonamidothioates gave products of widely varying activity, i.e., from 4.5 to >500 µg/g. Acylation of S-methyl ethylphosphonamidothioate gave products of higher house fly toxicity than the corresponding S-ethyl derivatives (compare 25 with 26, 27 with 28, and 30 with 31) even though the parent S-ethyl ethylphosphonamidothioate was slightly more active than the S-methyl analog. S-Methyl N-propionyl-P-ethylphosphonamidothioate (27) with a house fly LD_{50} of 4.5 µg/g showed the highest activity of the acylated derivatives but it was more than 5-fold less active than the parent compound.

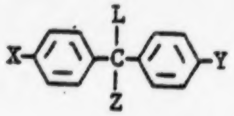
Although the acylated derivatives were poorer insecticides than the unacylated compounds, without exception acylation resulted in significant improvement in mammalian toxicity. None of the acylated derivatives was toxic to mice at 100 mg/kg, which was the highest dosage tested. In comparison, the mouse LD_{50} of 19 and 20, the parent phosphonamidothioate, was 16 mg/kg. In contrast to the acylated phosphonamidothioates, the single N-pyranyl derivative (35) was more toxic to mice than the parent phosphonamidothioate (15). This was not surprising owing to the greater lipophilicity of 35 and the likelihood of its reversion to the parent phosphonamidothioate in the acidic environment of the mouse's stomach. Since improvement in mammalian safety and insecticidal activity was not achieved, other N-pyranyl derivatives were not examined.

S-Methyl phosphonamidodithioate analogs were synthesized and examined for toxicological properties (36-39). While 36 and 37 were moderately toxic to house flies, they were less effective than the corresponding phosphonamidothioates (15 and 19). Substitution on the nitrogen atom also resulted in drastically reduced house fly toxicity.

Summary - A series of S-alkyl methyl- or ethylphosphonamidothioate esters analogous to methamidophos (O,S-dimethyl phosphoramidothioate) and acephate (O,S-dimethyl N-acetylphosphoramidothioate) were synthesized and evaluated for toxicological properties. Although S-methyl methyl- and ethylphosphonamidothioate were more effective against the house fly than methamidophos, the acylated derivatives showed variable toxicity but all were substantially less effective than acephate. The acylated derivatives, however, showed improved mouse toxicity. S-Methyl methyl- and ethylphosphonamidodithioate were less active against the house fly than methamidophos.

3. Insecticidal Activity of DDT Analogs.

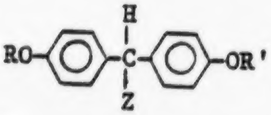
Introduction - In a previous report from this laboratory[3], a model for the hypothetical DDT receptor site was proposed to account for the variation in insecticidal activity of a series of DDT analogs. Briefly stated, the receptor was visualized as a cavity or pouch of approximately the same dimensions as DDT but of limited flexibility to accommodate unsymmetrical DDT analogs. Maximum perturbation of the nerve membrane was anticipated when maximum interaction between the DDT-type molecule and receptor site occurred, particularly with respect to the four key substituents X, Y, L and Z of the structure below.



For maximum interaction, the overall size of the DDT molecule (summation of the size of X, Y, L and Z) was crucial and any deviation from the optimum size resulted in reduced interaction and, therefore, reduced insecticidal activity. The model, within reasonable limits, provided the basis for the design of new DDT analogs and this section is concerned with the insecticidal properties of some of these compounds.

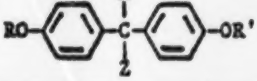
Toxicity - Data for the toxicity of the various DDT analogs which were synthesized and examined for insecticidal activity are presented in Tables 3 and 4. Analogs in which the bis-substituted phenyl moiety is attached to a terminal carbon atom are presented in Table 3 and those substituted in the 2-position are in Table 4.

2,2-Bis-(alkoxyphenyl)ethanes and 3,3-bis-(alkoxyphenyl)propanes - These compounds contain a hydrogen atom on the α-carbon atom and may be considered to be direct analogs of DDT. The model used for the design of these



compounds suggested that decrease in the size of Z could be compensated for by an increase in the size of the ring substituent (RO and R'O). Therefore, a series of analogs in which Z was kept at trifluoromethyl and one of the alkoxy ring substituents was increased in size were initially prepared. Owing to their biodegradable susceptibility[4], ring substituents were restricted to alkoxy moieties. To our disappointment, all of these compounds (40-48) were virtually devoid of insecticidal activity even though R' was increased in size to n-pentyl (R = methyl). Owing to the possibility that the trifluoromethyl moiety in the Z position was too small to interact properly with the DDT receptor site, an analogous series of compounds in which Z was maintained as pentafluoroethyl were prepared (49-54) but these also were insecticidally inactive. It is apparent from the toxicological data

TABLE 3. PHYSICAL AND INSECTICIDAL PROPERTIES OF DDT ANALOGS OF THE GENERAL STRUCTURE BELOW



Compd	R	R'	Z	House fly LD_{50}, μg/g	*Culex fatigans* LC_{50}, ppm
40	CH_3	CH_3	CF_3	>500	0.64
41	CH_3	C_2H_5	CF_3	>500	0.72
42	CH_3	*n*-C_3H_7	CF_3	>500	>1.0
43	CH_3	*i*-C_3H_7	CF_3	>500	>1.0
44	CH_3	*n*-C_4H_9	CF_3	>500	>1.0
45	CH_3	*n*-C_5H_{11}	CF_3	>500	>1.0
46	C_2H_5	C_2H_5	CF_3	>500	0.55
47	C_2H_5	*i*-C_3H_7	CF_3	>500	-
48	*i*-C_3H_7	*i*-C_3H_7	CF_3	>500	>1.0
49	CH_3	CH_3	C_2F_5	>500	>1.0
50	CH_3	C_2H_5	C_2F_5	>500	>1.0
51	CH_3	*n*-C_3H_7	C_2F_5	>500	>1.0

continued next page

TABLE 3. (cont'd)

Compd	R	R'	Z	House fly LD$_{50}$, µg/g	*Culex fatigans* LC$_{50}$, ppm
52	CH_3	*n*-C_4H_9	C_2F_5	>500	-
53	CH_3	*n*-C_5H_{11}	C_2F_5	>500	-
54	C_2H_5	C_2H_5	C_2H_5	>500	>1.0
55	CH_3	CH_3	CF_2Cl	55	0.07
56	C_2H_5	C_2H_5	CF_2Cl	48	0.066
57	*i*-C_3H_7	*i*-C_3H_7	CF_2Cl	123	0.27
58	*n*-C_3H_7	*n*-C_3H_7	CF_2Cl	96	0.18
59	*n*-C_4H_9	*n*-C_4H_9	CF_2Cl	210	>1.0

that analogs in which the substituent Z is totally substituted with fluorine atoms are inactive as insecticides. However, substitution of only one of the fluorine atoms by chlorine resulted in a notable increase in insecticidal activity (55-59) and highest activity was observed with 1-chloro-1,1-difluoro-bis-(p-ethoxyphenyl)ethane (56).

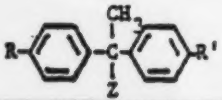
2,2-Bis-(p-substituted phenyl)-1,1-dihalopropanes - Because of the overall poor insecticidal activity observed with the perfluoro analogs and the apparent absence of compensating effects by the larger alkoxy ring substituents on the toxicity of these compounds, attention was turned to the 2,2-bis-(p-substituted phenyl)-1,1-dihalopropanes (cf. Table 4). These DDT analogs differ from those of Table 3 in that the hydrogen on the α-carbon atom is substituted by a methyl group. The rationale behind the examination of compounds of this type lies in the larger size of methyl compared to hydrogen and it was expected that reduction in the size of the Z moiety would be compensated for by the methyl group.

Toxicological data in Table 4 show that 64 is highly toxic to the test insects examined. Under the same bioassay conditions, the toxicity of DDT is 14 μg/g (LD_{50}) to house flies and 0.07 ppm (LC_{50}) to mosquito larvae and 64, therefore, is more effective to these insects than DDT. In a separate test against the Super Pollard resistant strain (R_{SP}) of house flies, the LD_{50} of 64 was determined to be 50 μg/g, a value which is at least 100-fold smaller than the >5000 μg/g reported for the LD_{50} of DDT to this strain[5]. The high tolerance of the R_{SP} strain to DDT is attributed to rapid detoxication of DDT to DDE by the action of DDT dehydrochlorinase. Compound 64, which cannot be dehydrochlorinated in the usual manner owing to the presence of the α-methyl group, therefore, would be expected to be toxic to this strain.

Except for the trifluoromethyl derivative (65), the other analogs also showed insecticidal activity but none were as potent as 64. The absence of insecticidal activity by 65 and 66 again points out the deactivating influence of the trifluoromethyl moiety in the Z position. Although the insecticidal activities of the compounds in Table 4 appear to be slightly less than their related DDT analogs, the results, nevertheless, indicate that derivatives which contain an α-methyl group also may possess high insecticidal activity. To our knowledge, this is the first case where significant insecticidal activity has been demonstrated with compounds of this type. It should be pointed out that the present study was exploratory in nature and only a handful of compounds were synthesized and evaluated. The approach, however, appears to be worthwhile and a large number of related derivatives remain to be examined.

Summary - A series of DDT analogs whose structures were based on a hypothetical model for the DDT receptor site were synthesized and examined for insecticidal activity. Several of these compounds showed insecticidal activity and one of them, 2,2-bis(p-ethoxyphenyl)-1,1-dichloropropane showed activity equal to that of DDT against house flies and mosquito larvae. This is the first case in which a DDT analog with an α-methyl moiety in place of hydrogen has been shown to have insecticidal activity.

TABLE 4. PHYSICAL AND INSECTICIDAL PROPERTIES OF DDT ANALOGS OF THE GENERAL STRUCTURE BELOW



Compd	R	R'	Z	House fly LD_{50}, μg/g	*Culex fatigans* LC_{50}, ppm
60	Cl	OCH_3	$CHCl_2$	72	0.115
61	Cl	OCH_3	CHClBr	68	0.145
62	Br	OCH_3	$CHCl_2$	87	0.23
63	Br	OCH_3	CHBrCl	98	0.18
64	OC_2H_5	OC_2H_5	$CHCl_2$	10	0.06
65	OCH_3	OCH_3	CF_3	>500	>1.0
66	OPr-*n*	OPr-*n*	CF_3	>500	>1.0
67	OCH_3	OPr-*n*	CF_2Cl	>500	>1.0

4. Effect of Ring Substituents on the Inhibition of Cholinesterase by Carbamate Esters.

Introduction - In a previous report[6], we described a number of potent *N*-hydroxy- and *N*-methoxy-*N*-methylcarbamate esters which proved to behave completely as reversible, competitive inhibitors of AChE. These esters, which differ from the methylcarbamates by replacement of the nitrogen proton with a methoxy or hydroxy moiety, undoubtedly inhibit AChE by complexing with the enzyme active site. Owing to the reversible nature of inhibition by these esters, it was evident that determination of the equilibrium constant for enzyme-inhibitor complex (K_i) would provide a useful measure of the effect of ring substituents on AChE binding with carbamate esters. This section is concerned with the inhibition of three different cholinesterases by substituted phenyl *N*-methoxy-*N*-methyl-carbamates and analysis of the data to assess the effect of ring substituents on binding.

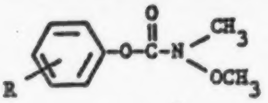
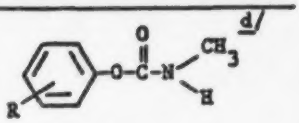
Anticholinesterase Activity - Values for K_i, the dissociation constant for reversible, competitive inhibition of HFAChE, BAChE, and HSChE by the various substituted aryl *N*-methoxy-*N*-methylcarbamates, are presented in Table 5.

Although all of the *N*-methoxy-*N*-methylcarbamates behaved as reversible inhibitors of these cholinesterases, a number of compounds showed mixed competitive inhibition, particularly with HFAChE. Equilibrium constants were not obtained in the cases where mixed inhibition was observed.

Of the three cholinesterases, HFAChE proved to have the greatest sensitivity to changes in the ring substituent. The range of K_i was approximately 10^{-4} fold, from 9.94×10^{-9}M for the 3-trimethylammonium derivative (XV) to 1.38×10^{-4}M for the 4-nitro derivative (XXII). Of the 27 *N*-methoxy-*N*-methylcarbamates examined, nine showed mixed competitive kinetics and one, the 2-*t*-butyl derivative (V), was too inactive and insoluble to obtain a finite K_i value. On an overall basis, derivatives with substituents in the *ortho* and *meta* positions were virtually equal in inhibiting HFAChE (cf. equation 2). However, methoxycarbamates with substituents in both *meta* positions (XXIV and XXV) were exceptionally potent inhibitors of HFAChE, reminiscent of the high anticholinesterase activities of the corresponding methylcarbamate esters[7]. Methoxycarbamates with *para* substituents were poorest against all three cholinesterases.

Reversible, competitive kinetics were observed for all compounds with BAChE. As in the case of HFAChE, the 3-trimethylammonium derivative, with a K_i of 1.35×10^{-7}M, was the most potent inhibitor and was about 10^{3}-fold stronger than the weakest inhibitor (XX). It was, however, 10-fold less effective against BAChE than HFAChE. Inhibition of BAChE by the methoxycarbamates differed from that of HFAChE in other aspects. On the whole, methoxycarbamates substituted in the *ortho* position were significantly stronger inhibitors of BAChE than the *meta*-substituted analogs. This suggests that the distance between the sites in BAChE which interact with the ring substituent and carbamate moiety to form the enzyme-inhibitor complex is smaller than that for HFAChE.

TABLE 5. DISSOCIATION CONSTANTS, K_i, FOR THE COMPETITIVE REVERSIBLE INHIBITION OF THREE CHOLINESTERASES BY SUBSTITUTED ARYL N-METHOXY-N-METHYLCARBAMATES. INCLUDED ARE HOUSE FLY TOXICITIES AND HFAChE I_{50} VALUES FOR CORRESPONDING SUBSTITUTED ARYL METHYLCARBAMATES

					 d/
			K_i X 10^5 M		
No.	R	HFAChE a/	BAChE b/	HSChE c/	I_{50} X 10^5 HFAChE
I	H	mixed	3.48	mixed	20
II	$2\text{-}CH_3$	mixed	2.17	4.87	14
III	$2\text{-}C_2H_5$	0.650	0.453	5.52	1.3
IV	$2\text{-}\underline{i}\text{-}C_3H_7$	0.372	0.156	0.376	0.6
V	$2\text{-}\underline{t}\text{-}C_4H_9$	>100	3.91	1.22	0.6
VI	2-Cl	0.311	0.193	0.254	0.5
VII	2-Br	0.0752	0.110	0.261	0.22
VIII	$3\text{-}CH_3$	mixed	2.14	2.34	1.4
IX	$3\text{-}C_2H_5$	mixed	1.59	0.290	0.48
X	$3\text{-}\underline{i}\text{-}C_3H_7$	0.0872	0.134	0.0500	0.034
XI	$3\text{-}\underline{t}\text{-}C_4H_9$	0.0681	0.206	0.147	0.04
XII	3-Cl	0.951	0.796	0.999	5.0
XIII	3-Br	mixed	0.827	0.905	1.3
XIV	$3\text{-}N(CH_3)_2$	mixed	1.44	0.349	0.8
XV	$3\text{-}\overset{+}{N}(CH_3)_3I^-$	0.000994	0.0135	0.0421	0.0018
XVI	$4\text{-}CH_3$	4.22	12.6	2.93	10

continued next page

TABLE 5. (cont'd)

XVII	4-C_2H_5	4.95	12.9	5.88	3.8
XVIII	4-*i*-C_3H_7	1.87	6.01	9.68	7.0
XIX	4-*t*-C_4H_9	1.69	3.01	3.71	15
XX	4-Cl	mixed	17.3	6.25	24
XXI	4-Br	5.27	6.56	4.25	10
XXII	4-NO_2	13.8	16.1	42.0	300
XXIII	3,5-di-CH_3	mixed	0.187	0.485	0.6
XXIV	3,5-di-*i*-C_3H_7	0.00294	0.132	0.0252	0.0033
XXV	3,5-di-*t*-C_4H_9	0.00367	0.369	0.0866	0.0078
XXVI	1-Naphthyl	mixed	0.187	0.0735	0.09
XXVII	carbofuran	0.0168	0.0462	0.0527	0.025

[a] K_m (acetyl thiocholine) 4.34 (0.32) X 10^{-5} *M*, average standard error for the determinations of K_m and K_p is 7.6%.

[b] K_m (acetyl thiocholine) 1.23 (0.11) X 10^{-4} *M*, average standard error for the determinations of K_m and K_p is 7.9%.

[c] K_m (butyryl thiocholine) 4.01 (0.33) X 10^{-4} *M*, average standard error for the determinations of K_m and K_p is 10.9%.

[d] Data for aryl methylcarbamates are from reference 7.

Competitive, reversible kinetics were observed for HSChE, a pseudocholinesterase, with all methoxycarbamates except the unsubstituted derivative (I). It is noteworthy that the charged 3-trimethylammonium derivative (XV) was not the most effective inhibitor and highest activity was observed with the 3,5-di-*i*-propyl analog (XXIV). The overall range in K_i was about the same as that found for BAChE.

Analysis - In the analysis of the effect of ring substituents on anticholinesterase activity of the aryl *N*-methoxy-*N*-methylcarbamates, the relationship between K_i and anticholinesterase activity (I_{50}) of the corresponding aryl methylcarbamates was first examined. An excellent linear relationship between these parameters ($r = 0.97$) was obtained, indicating that ring substituents in both series of compounds interact with the enzyme active site in a similar manner.

Insight into the nature of the interaction between the ring substituent of the methoxycarbamates and enzyme active site was obtained by examining (by means of multiple regression analysis) the relation between $-\log K_i$ and the free energy parameters π, σ and E_s. These substituent constants are representative parameters for estimation of hydrophobic (π), electronic (σ), and steric (E_s) effects. Satisfactory correlation was obtained with π and the ring position terms d_1 and d_2 according to the equation shown below, where $d_1 = 1$ and $d_2 = -1$ for *ortho*-substituted compounds, $d_1 = d_2 = 1$ for *meta*-substituted compounds and $d_1 = -2$ and $d_2 = 0$ for *para*-substituted compounds. Correlation, however, was poor when π was used alone, e.g., $r = 0.31$ for BAChE.

BAChE

$$-\log K_i = \underset{(0.284)}{4.323} + \underset{(0.284)}{0.663}\,\pi + \underset{(0.102)}{0.455}\,d_1 - \underset{(0.180)}{0.275}\,d_2 \qquad (1)$$

$n = 20 \qquad r = 0.93 \qquad s = 0.30$

HFAChE

$$-\log K_i = \underset{(0.572)}{4.668} + \underset{(0.493)}{0.529}\,\pi + \underset{(0.147)}{0.424}\,d_1 \qquad (2)$$

$n = 13 \qquad r = 0.92 \qquad s = 0.35$

HSChE

$$-\log K_i = \underset{(0.550)}{4.447} + \underset{(0.541)}{0.463}\,\pi\ \underset{(0.175)}{0.382}\,d_1 + \underset{(0.328)}{0.104}\,d_2 \qquad (3)$$

$n = 19 \qquad r = 0.79 \qquad s = 0.51$

n is the number of compounds and *s* is the standard error. Parenthetical values are 95% confidence intervals for the intercept and coefficients. Neither σ nor E_s had any effect in improving the correlations represented by the equations and, further, poorer correlation was observed when each of these substituents was used alone with the position terms. Based on *t* values, the position parameter d_1 was of greatest significance with all three enzymes, followed in order by π and d_2. In the case of BAChE, all parameters were significant at the 99% level. The importance of the position parameters in the regression analysis is shown graphically in Figs. 2 and 3. Figure 2 provides a plot of $-\log K_i$ for BAChE against π alone, while Fig. 3 includes the position terms according to equation 1. It is evident from Fig. 2 that within each series of compounds, i.e., *ortho*-, *meta*-, or *para*-substituted methoxycarbamates, a reasonably good linear relationship between $-\log K_i$ and π is obtained and, also, that the *ortho* derivatives are intrinsically more active than the *meta* which in turn are more active than the *para* derivatives. The position terms, therefore, were necessary to compensate for differences in intrinsic activities of the three series of compounds.

The values for d_1 and d_2 for the three ring positions reveal an order for the inhibition of *ortho* > *meta* > *para* for BAChE, *ortho* > *meta* > *para* for HFAChE and *meta* > *ortho* > *para* for HSChE. The heavy dependence of the correlation on ring position strongly suggests that the substituents interact with a lipophilic site situated at a fixed distance (about 0.4 nm) from the esteratic site, i.e., the site which binds with the carbamate moiety.

The presence of π in the regression equations, of course, is indicative of the involvement of hydrophobic bonding in the interaction between the ring substituent and lipophilic site. Evidently, the distance between the lipophilic site and esteratic site is larger for HSChE than for either BAChE or HFAChE.

Overall, it is apparent from the regression analyses that hydrophobic bonding and position of the substituent on the phenyl ring are of greatest significance in explaining the variation in anticholinesterase activities of the substituted phenyl *N*-methoxy-*N*-methylcarbamates examined in this study. The results imply that reversible binding of the methoxycarbamates to the enzyme active site occurs predominantly through hydrophobic interaction. The more or less direct relationship observed between $-\log K_i$ for the methoxycarbamates and different estimates of anticholinesterase activity of methylcarbamates, e.g., $-\log I_{50}$ and $\log k_i$, strongly suggests a major role for hydrophobic bonding in the affinity of methylcarbamates for AChE.

Summary - A series of 27 substituted aryl *N*-methoxy-*N*-methylcarbamates were synthesized and their ability to reversibly inhibit house fly-head and bovine erythrocyte acetylcholinesterase, and horse serum cholinesterase was determined. These compounds were all competitive, reversible inhibitors of bovine erythrocyte acetylcholinesterase but some of them showed mixed competitive inhibition against the house fly-head and horse serum enzymes. Dissociation constants (K_i) as small as 9.9×10^{-9} *M* and as large as 1.4×10^{-4} *M* were observed. A highly satisfactory correlation between $\log K_i$ for the inhibition of fly-head acetylcholinesterase by the *N*-methoxy-*N*-methylcarbamates and $-\log I_{50}$ for the inhibition of the same enzyme by the corresponding methylcarbamates was noted. Analysis of the anticholinesterase data by multiple regression showed $-\log K_i$ to be related to Hansch's π constant and ring position terms. The results indicate that reversible

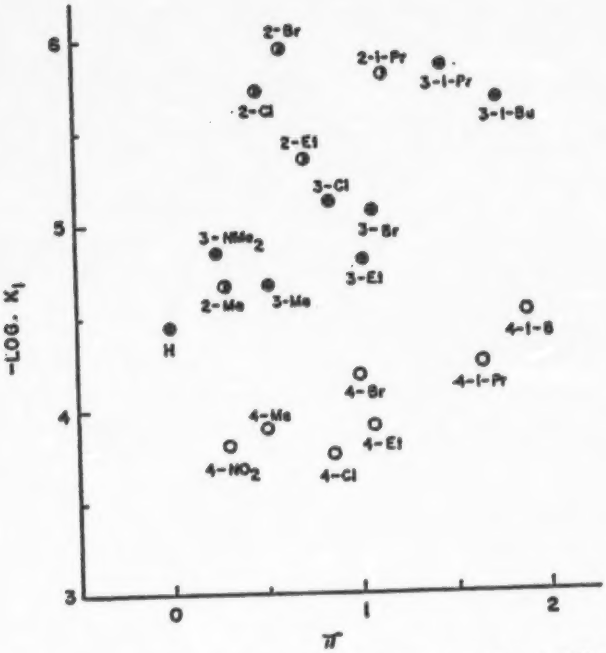


Fig. 2. Relation between -log K_I for the inhibition of BAChE by substituted phenyl N-methoxy-N-methylcarbamates and Hansch's π constant.

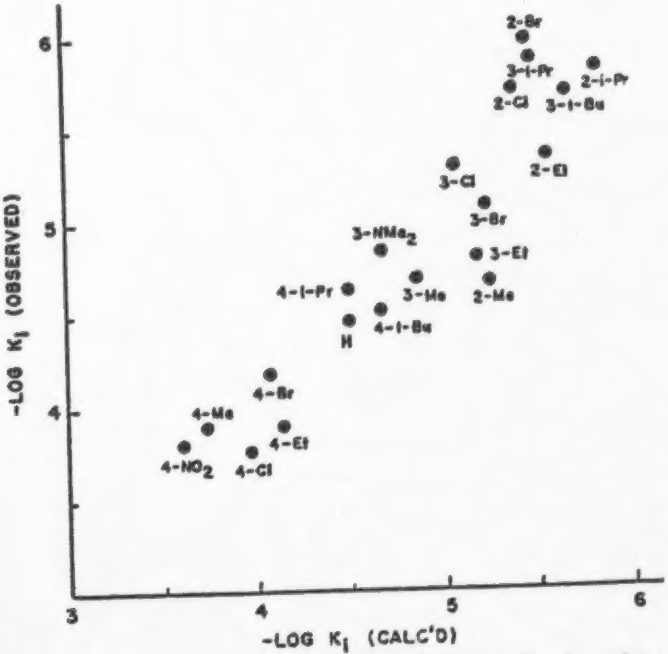


Fig. 3. Relation between observed -log K_I for the inhibition of BAChE by substituted phenyl N-methoxy-N-methylcarbamates and -log K_I calculated according to equation 1.

binding of these compounds to acetylcholinesterase occurs by hydrophobic bonding.

5. References.

1. Taft, R.W., Jr., in "Steric Effects in Organic Chemistry", Newman, M.S., Ed., Wiley, New York, N.Y., 1956, p. 556.
2. Quistad, G.B., T.R. Fukuto, R.L. Metcalf, J. Agr. Food Chem. 18, 189 (1970).
3. Fahmy, M.A.H., T.R. Fukuto, R.L. Metcalf, R.L. Holmstead, J. Agr. Food Chem. 21, 585 (1973).
4. Kapoor, I.D., R.L. Metcalf, A.S. Hirwe, P.Y. Lu, J.R. Coats, R.F. Nystrom, J. Agr. Food Chem. 20; 1 (1972).
5. Metcalf, R.L., T.R. Fukuto, Bull. Wld. Hlth. Org. 38, 633 (1968).
6. Chiu, Y.C., M.A.H. Fahmy, T.R. Fukuto, Pestic. Biochem. Physiol. 3, 1 (1973).
7. Metcalf, R.L., Bull. Wld. Hlth. Org. 44, 43 (1971).

D. BIOLOGICAL AND ENVIRONMENTAL ALTERATION OF INSECTICIDES

1. Characterization of the Bound Phenthoate Residues in Citrus.

Introduction - Phenthoate or O,O-dimethyl S-[α-(carboethoxy)benzyl] phosphorodithioate (Cidial®) (I) is an effective wide spectrum organophosphorus insecticide of low mammalian toxicity. It is approximately 950-fold more toxic to the common house fly (LD_{50} 5 mg/kg) than to the rat LD_{50} (4728 mg/kg)[1]. The proven usefulness of phenthoate in controlling citrus scale insects prompted a study of its fate and environmental alteration in and on citrus trees[2]. The products recovered from citrus fruit extracts were phenthoate and its metabolites. Approximately 22 to 35% of the applied radioactivity present in the peel was unextractable with acetone and was considered to be bound but was not characterized. The metabolism and fate of phenthoate in Valencia orange fruits were reexamined with emphasis on the characterization of bound phenthoate residues in the fruit peel.

Results - Data for the distribution of radioactivity in the surface wash, peel, and pulp after treatment of orange fruit with ^{14}C-phenthoate are presented in Table 1. The amount of radioactivity applied to each fruit varied because of their size and nonuniformity in brush application. The data show a gradual decline in external fruit surface wash with a gradual increase in the percentage of radioactivity recovered in the internal acetone extract and peel residue. For example, more than 42.6% of the radioactivity was recovered from the peel residue on or after 14 days after treatment of fruits. Less than 1.0% of the radioactivity was recovered in the fruit pulp at any time interval.

Data indicating the nature and distribution of metabolites recovered and identified from the different fractions of orange fruit are presented in Tables 2, 3, and 4. Five principal metabolites, identified as phenthoate oxon (II), demethyl phenthoate (III), phenthoate acid (IV), ethyl mandelate (V), mandelic acid (VI), along with unchanged phenthoate (I) were obtained. The major compound isolated from the external fruit surface wash was unchanged I followed by metabolite V (Table 2), with a gradual decline in the amounts of I and a gradual increase in V with time. The major product recovered from the internal acetone extract of the fruit peel was I, followed by V, IV, VI, and III (Table 3). The major conjugated metabolites isolated from the peel residue were ethyl mandelate (V) and mandelic acid (VI) followed by metabolites III, and IV. Phenthoate oxon (II) was not detected as a conjugate in the peel residue. The unextractable radioactivity from aqueous phase and peel residue was not identified (Table 4).

Summary - Previous work from this laboratory reported phenthoate oxon (II), demethyl phenthoate (III), mandelic acid (VI) and bis-[α-(carboethoxy)-benzyl] disulfide as metabolites of phenthoate in orange fruit[2], but no attempt was made to isolate or identify the nature of the bound radioactivity in the fruit peel residue. In this study, special attention was given to the identification of the bound residues in the fruit peel (Table 4). β-Glucosidase, hydrochloric acid, and sodium hydroxide solubilized

TABLE 1. THE PENETRATION OF ^{14}C-PHENTHOATE INTO ORANGE FRUIT, AND THE DISTRIBUTION OF RADIOACTIVITY IN THE SURFACE WASH, PEEL, AND PULP.[a]

Radioactivity found in substrate	Percentage of recovered radioactivity (days) 0	3	7	14	28	35
External fruit surface wash	98.2	76.8	70.5	31.5	11.7	8.4
Fruit peel						
Internal acetone extract	1.0	10.3	13.7	25.3	29.5	29.1
Peel residue after extraction	0.8	12.9	15.3	42.6	58.0	61.7
Fruit pulp	0	0	0.5	0.6	0.8	0.8
Total recovered	100.0	100.0	100.0	100.0	100.0	100.0

[a] Number of fruits sampled at 0, 3, 7, 14, 28, and 35 days = 5, 6, 7, 7, 10 and 10, respectively.

TABLE 2. AMOUNT OF ^{14}C METABOLITES BOUND IN THE ORANGE FRUIT EXTERNAL SURFACE WASH AT DIFFERENT TIME INTERVALS AFTER APPLICATION

	Percentage of recovered radioactivity (days)					
Compound found	0	3	7	14	28	35
I. phenthoate	95.7	95.8	61.5	50.5	87.5	55.4
II. phenthoate oxon	0	0	0	0.6	0	0
III. demethyl phenthoate	0	0.1	3.3	1.1	1.2	7.0
IV. phenthoate acid	0.6	0.2	1.3	0.9	6.5	1.3
V. ethyl mandelate	1.9	2.9	24.7	36.8	0	21.8
VI. mandelic acid	0	0	0	5.4	1.2	4.8
Others (unidentified)	2.0	1.0	9.1	4.5	3.1	9.7
Total	100.2	100.0	99.9	99.8	99.5	100.0

TABLE 3. AMOUNT OF ^{14}C METABOLITES BOUND IN THE ORANGE FRUIT PEEL INTERNAL ACETONE EXTRACT AT INDICATED TIME INTERVALS AFTER APPLICATION

Compound found	Percentage of recovered radioactivity (days)				
	3	7	14	28	35
I. phenthoate	60.8	86.5	79.7	55.9	76.5
III. demethyl phenthoate	1.9	0.8	4.2	3.6	1.3
IV. phenthoate acid	5.7	1.6	11.9	17.0	0.3
V. ethyl mandelate	26.8	9.3	0.7	13.8	13.6
VI. mandelic acid	3.2	0	0	4.1	6.1
Others (unidentified)	1.6	1.8	3.5	5.6	2.2
Total	100.0	100.0	100.0	100.0	100.0

TABLE 4. AMOUNT OF ^{14}C METABOLITES BOUND IN THE ORANGE FRUIT PEEL RESIDUE AT INDICATED TIME INTERVALS AFTER APPLICATION

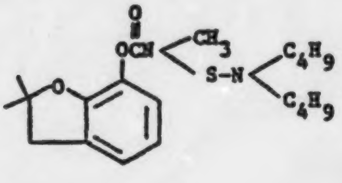
	Percentage of recovered radioactivity (days)				
Compound found	3	7	14	28	35
III. demethyl phenthoate	0	18.4	5.2	9.8	4.6
IV. phenthoate acid	4.7	0	3.9	2.9	0
V. ethyl mandelate	52.8	47.3	46.8	36.5	48.5
VI. mandelic acid	34.0	30.8	34.6	39.9	37.4
Others (unidentified)	2.3	1.1	2.1	3.6	1.6
Unextractables from aqueous phase	2.6	1.2	3.2	4.2	2.2
Unextractables from peel residue	3.6	1.2	4.2	3.1	5.7
Total	100.0	100.0	100.0	100.0	100.0

about 92 to 98% of the bound radiocarbon from the insoluble residue, which implies that the components of this residue were enzyme, acid and base labile. The conjugated metabolites were hydrolytic degradative products of phenthoate. The various alteration and conjugated products which were isolated and identified represent compounds which would normally be expected from the structure of phenthoate. In terms of actual amounts (calculated as phenthoate equivalents), the levels of bound radioactivity in the fruit peel based on the dry weight of the peel were 0.01, 0.16, 0.08, 0.24, 0.25 and 0.19 ppm at 0, 3, 7, 14, 28 and 35 days, respectively, after treatment. At 35 days, the calculated amounts of ethyl mandelate (V) and mandelic acid (VI), the principal metabolites bound as peel residue, were 0.05 and 0.03 ppm, respectively. All of the alteration products of phenthoate may be regarded as detoxication products.

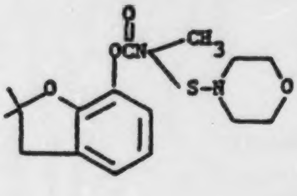
2. Metabolism of Dibutylaminosulfenyl and Morpholinosulfenyl Carbofuran in Plants, Animals and Soil.

a. Metabolism in Corn and Cotton Plants.

Introduction - 2,3-Dihydro-2,2-dimethyl-7-benzofuranyl (di-*n*-butylaminosulfenyl)(methyl)carbamate (DBSC or dibutylaminosulfenyl carbofuran) and 2,3-dihydro-2,2-dimethyl-7-benzofuranyl (morpholinosulfenyl)(methyl)carbamate (MSC or morpholinosulfenyl carbofuran) are currently undergoing development as potentially useful insecticides.



DBSC



MSC

Compared to carbofuran, DBSC, with an oral LD_{50} of about 100 mg/kg, is 12-fold less toxic to rats (female) and is more effective against some insects. MSC is at least 5-fold less toxic to the white mouse than carbofuran. In order to assess potential hazards arising from their use as agricultural chemicals, a study was conducted on the metabolic fate of DBSC and MSC in plants.

Abbreviations - The following abbreviations were used for the different compounds used in this study.

Compound	Abbreviation
Bis-carbofuran-N,N-disulfide	Disulfide
Dibutylaminosulfenyl carbofuran	DBSC
Morpholinosulfenyl carbofuran	MSC
Carbofuran phenol	Phenol
3-Keto-carbofuran phenol	3-C=O-P
3-Hydroxy-carbofuran phenol	3-OH-P
Carbofuran	--
3-Keto-carbofuran	3-C=O-CF
3-Hydroxy-carbofuran	3-OH-CF
N-Hydroxymethyl carbofuran	N-CH_2OH-CF
N-Desmethyl-carbofuran	NH_2-CF
3-Keto-N-hydroxymethyl-carbofuran	3-C=O-N-CH_2OH-CF
3-Keto-N-desmethyl-carbofuran	3-C=O-NH_2-CF
3-Hydroxy-N-hydroxymethyl-carbofuran	3-OH-N-CH_2OH-CF
3-Hydroxy-N-desmethyl-carbofuran	3-OH-NH_2-CF

Metabolism of DBSC in Cotton Plants following Stem Injection - DBSC metabolites in cotton plants were identified 1, 3, 6, and 10 days following stem injection of ring-^{14}C-DBSC (6.57 μg/plant) or carbonyl-^{14}C-DBSC (5.82 μg/plant).

Table 5 summarizes data for the distribution, identity, and relative amounts of the various metabolites isolated from the cotton plant after treatment with ring-^{14}C-DBSC. The identity of the various metabolites was confirmed by cochromatography in at least 4 different solvent systems and also by two dimensional cochromatography. Total recoveries of injected radioactivity from the 1-, 3-, 6-, and 10-day plants were 76.9, 82.3, 91.5, and 82.4%, respectively. After 1 day, most of the radioactivity (90.4%) was observed as organo-soluble material and only 8.5% was present in water-solubles, presumably as conjugated compounds. However, the amount of conjugate increased during the experimental period and reached 40.4% after 10 days.

The cotton plant was effective in metabolizing DBSC. After 1 day, only 42.4% of the recovered radioactivity was present as the parent carbamate, and this gradually decreased during the 10-day test period. The principal metabolite after 1 and 3 days was carbofuran but 3-OH-CF (free plus conjugate) was of equal importance at 6 and 10 days. During the 10-day test

TABLE 5. METABOLISM OF ^{14}C-DIBUTYLAMINOSULFENYL CARBOFURAN (RING AND CARBONYL LABELS) IN COTTON PLANTS AFTER STEM INJECTION [a]

Metabolites	Percent of Total DBSC Applied (Days)								
	Ring label				Carbonyl label				
	1	3	6	10	0	1	3	6	10
Total recovery	76.9	82.3	91.5	82.4	~ 100	78.9	77.0	90.6	77.5
A. Organo-soluble									
DBSC	42.4	32.1	28.0	27.8	92.8	53.1	35.4	26.6	27.7
Unknown	0	0	0	0	0	0	1.2	0	0
3-OH-P	1.4	1.1	0.9	1.5	-	-	-	-	-
Carbofuran	36.1	28.2	15.6	20.0	4.6	28.8	30.8	17.6	21.6
3-C=O-CF	2.1	2.0	2.2	1.5	0	0.8	1.4	1.8	1.2
3-OH-CF	7.5	9.6	8.7	3.6	0	5.7	6.3	6.0	2.7
_N_-CH$_2$OH-CF	0.3	1.6	1.8	0.2	0	1.3	2.1	2.7	1.2
Unknown II	0.6	0.6	1.0	0.4	2.0	0.3	0.2	0.2	0.3
Sub-total	90.4	75.2	58.2	55.0	99.4	90.0	77.4	54.9	54.7
B. Water-soluble conjugate									
Phenol	0.2	0.9	1.8	1.9	-	-	-	-	-
3-C=O-P	0.1	1.6	4.9	7.2	-	-	-	-	-
3-OH-P	0.2	0.2	1.2	1.0	-	-	-	-	-
Carbofuran	3.2	2.1	1.1	0.8	-	2.2	1.7	0.5	0.9
NH$_2$CF[b]	0.5	2.4	3.1	4.2	-	0.1	0.2	0.4	0.6
3-OH-CF	2.4	11.5	18.7	16.8	-	5.4	14.8	33.5	29.1
Unknown I	0.1	0.6	0.5	0.5	-	<0.1	<0.1	0.1	0.1
3-OH-NH$_2$-CF[b]	0.2	0.6	1.4	1.1	-	0.1	0.1	0.3	0.2
Unknown II	0.1	0.2	0.5	0.3	-	0.2	0.3	0.7	0.7
Unextractable	1.5	2.5	5.2	6.6	-	1.3	3.2	6.4	7.9
Sub-total	8.5	22.6	38.4	40.4	0.6	9.3	20.3	41.9	39.5
C. Unextractable (Radioactivity in straw)	1.3	2.3	3.6	4.6	0.1	0.8	2.4	3.3	5.7
Total	100.2	100.1	100.2	100.0	100.1	100.1	100.1	100.1	99.9

[a] One microliter of an ethanol-acetone (1:1) solution containing ring-^{14}C-DBSC (6.57 μg, 0.201 μci) or carbonyl-^{14}C-DBSC (5.82 μg, 0.219 μci) was injected into the stem of each cotton plant (1.23 g and 1.19 g in fresh weight, respectively) in the trifoliate stage. There were 4 plants per set.

[b] NH$_2$-CF and 3-OH-NH$_2$-CF are artificial products from the acid-hydrolysis treatment and actual metabolites are _N_-CH$_2$OH-CF and 3-OH-_N_-CH$_2$OH-CF, respectively.

period, the amount of carbofuran gradually decreased with more or less commensurate increase in total 3-OH-CF. Conjugated 3-C=O-P and NH_2-CF also increased rapidly during the test period. Of the unknowns, I did not correspond to either 3-C=O-N-CH_2OH-CF or 3-C=O-NH_2-CF, although its R_f value was very close to that of 3-C=O-N-CH_2OH-CF. Unknown II represents the radioactive material at or near the origin spot of the TLC chromatogram. The unhydrolyzed water-soluble conjugates (unextractable) of the 1-, 3-, and 10-day samples were subjected to a second hydrolysis by heating in 0.5 N hydrochloric acid for 1 hr. About 20-35% of the unhydrolyzed conjugates were changed into dichloromethane-extractable compounds. The major products were 3-OH-NH_2-CF and unknown I, along with small amounts of NH_2-CF and unknown II.

The metabolites indicated as NH_2-CF and 3-OH-NH_2-CF doubtlessly are conversion products from the respective N-hydroxymethyl derivatives during acid hydrolysis. This was confirmed by subjecting the water-soluble conjugate to β-glucosidase hydrolysis and analysis by two dimensional TLC. Thin-layer chromatograms obtained after acid hydrolysis and enzymic hydrolysis clearly indicate the absence of NH_2-CF and 3-OH-NH_2-CF and the presence of N-CH_2OH-CF and 3-OH-N-CH_2OH-CF after enzymic hydrolysis. This finding clearly indicates that NH_2-CF and 3-OH-NH_2-CF were produced by the acid-hydrolysis treatment.

The results with ring-^{14}C-DBSC were supported by identical experiments using carbonyl-^{14}C-DBSC. Total recoveries of the injected radioactivity from 1-, 3-, 6-, and 10-day plants were 78.9, 77.0, 90.6 and 77.5%, respectively. The distribution of radioactivity between organo-soluble and water-soluble conjugates, the types of metabolites and their relative amounts were almost the same as those found with the ring label except for the obvious absence of the phenols. The metabolites of greatest magnitude were carbofuran after 1 and 3 days and 3-OH-CF (free plus conjugate) after 6 and 10 days.

Metabolism in cotton plants following stem injection at the higher dose of 25.0 μg/plant carbonyl-^{14}C-DBSC also was studied. The metabolites found were the same as at the lower dose except for the detection of very small amounts of 2 additional unknown compounds (both R_f values are higher than that of DBSC). However, differences in the relative amounts of organo-soluble and water-soluble conjugates, and relative amounts of metabolites were noted. After 3 days, 89.0% of the recovered radioactivity was isolated as organo-soluble material and 10.0% as conjugates. The percentage of conjugates increased to 25.5% after 10 days, but 70.8% of the radioactivity was still present as organo-solubles. The metabolite of greatest magnitude at both time intervals was carbofuran followed by 3-OH-CF.

Metabolism of MSC - The metabolism of MSC in cotton plants was determined following injection of 4.22 μg/plant carbonyl-^{14}C-MSC into the stem. Table 6 summarizes data for the distribution, identity, and relative amounts of the various metabolites found at the indicated time intervals after treatment. Overall, the results were similar to those found in the metabolism of carbonyl labeled DBSC. Most of the MSC was converted to

TABLE 6. METABOLISM OF ^{14}C-MORPHOLINOSULFENYL CARBOFURAN (CARBONYL LABEL) IN COTTON PLANTS AFTER STEM INJECTION.[a]

Metabolites	Percent of Total MSC Applied (Days)			
	1	3	6	10
Total recovery	87.2	87.7	71.9	84.5
A. Organo-soluble				
MSC	4.8	7.0	7.5	4.4
Carbofuran	67.4	43.8	27.3	18.9
3-C=O-CF	2.5	4.4	4.1	4.5
3-OH-CF	16.5	19.0	19.9	18.8
Unknown II	0.1	0.3	0.5	0.5
Sub-total	91.3	74.5	59.3	47.1
B. Water-soluble conjugate				
Carbofuran	0.8	0.6	1.3	0
NH_2-CF[b]	0.6	2.6	4.4	6.8
3-OH-CF	3.3	12.7	20.8	30.0
Unknown I	0.1	0.7	1.0	0.9
3-OH-NH_2-CF[b]	0.2	0.4	0.4	0.5
Unknown II	<0.1	0.1	0.2	0.4
Unextractable	2.2	5.8	7.6	7.7
Sub-total	7.2	22.9	35.7	46.3
C. Unextractable (Radioactivity in straw)	1.6	2.7	5.1	6.7
Total	100.1	100.1	100.1	100.1

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[a] One microliter of an ethanol-acetone (1:1) solution containing carbonyl-^{14}C-MSC (4.22 μg, 0.179 μci) was injected into the stem of each cotton plant (0.929 in fresh weight) in the early trifoliate stage. There were 4 plants per set.

[b] NH_2-CF and 3-OH-NH_2-CF are artificial products from the acid-hydrolysis treatment and actual metabolites are N-CH_2OH-CF and 3-OH-N-CH_2OH-CF, respectively.

carbofuran within 1 day and the metabolites of greatest magnitude were carbofuran after 1 and 3 days and 3-OH-CF (free plus conjugate) after 6 and 10 days. As observed in the metabolism of DBSC, a marked increase in conjugated 3-OH-CF was also observed after 3 to 10 days. The amount indicated for 3-OH-CF in Table 6 probably includes small amounts of N-CH_2OH-CF since clean separation of 3-OH-CF from N-CH_2OH-CF by TLC was not accomplished. The TLC properties of unknown I corresponded to unknown I obtained with DBSC.

Metabolism of DBSC on and in Cotton Plants After Foliage Treatment - DBSC metabolites in cotton plants were identified after painting carbonyl and ring labeled DBSC on the leaves. Table 7 summarizes data for the distribution, identity and relative amounts of the various metabolites 1, 3, 6, and 10 days after treatment. Total recoveries of painted DBSC were in the range of 77-94%.

Data for the carbonyl labeled material show that at 1 day most of the radioactivity (69.3%) was observed in the leaf-rinse while 18.9 and 11.0% were found internally as organo-soluble and water-soluble conjugate fractions, respectively. However, the percentage of leaf-rinse decreased rapidly during the test period and was 15.3% after 10 days, along with a commensurate increase in radioactivity for the water-soluble conjugate (66.4% after 10 days). The same metabolites as those identified following stem injection were observed and the major metabolites were carbofuran after 1 day and 3-OH-CF (free plus conjugate) after 3, 6, and 10 days. Overall, the rate of metabolism after foliage treatment appeared to be more rapid than after stem injection.

The same metabolites found in the internal extract were also observed in the leaf-rinse. The leaf-rinse, obtained by washing the leaves with benzene-dichloromethane (1:1), showed no color attributable to plant pigments and the relative amounts of the metabolites in the leaf-rinse were quite different from those found in the organo-soluble fraction. These results suggest that the metabolites in the leaf-rinse are actually those present on the surface and were not leached from the leaf by the solvent.

Results obtained using carbonyl labeled DBSC were substantiated by identical experiments using ring labeled material. The distribution, identity, and amount of radioactivity in the leaf-rinse, organo-soluble, and water-soluble fractions were almost the same as those found with the carbonyl labeled material.

Metabolism of DBSC and MSC in Corn Plants following Stem Injection - Table 8 summarizes data for the distribution, identity and relative amounts of the various metabolites of DBSC in corn plants following stem injection of 5.36 μg carbonyl labeled DBSC. Total recoveries at 7 and 10 days were poor (49.8 and 45.6%, respectively) and suggest loss of radioactivity by carbon dioxide evolution. The same metabolites found in cotton were also found in corn, the major metabolites being carbofuran and 3-OH-CF. Differences

TABLE 7. METABOLISM OF ^{14}C-DIBUTYLAMINOSULFENYL CARBOFURAN (RING AND CARBONYL LABELS) ON AND IN COTTON PLANTS AFTER FOLIAGE TREATMENT.[a]

Metabolites	Percent of Total DBSC Applied (Days)					
	Ring label		Carbonyl label			
	3	10	1	3	6	10
Total recovery	82.6	84.1	94.0	82.6	77.0	83.5
A. External extract (leaf-rinse)						
DBSC	4.7	0.8	18.1	4.9	3.1	1.6
Unknown	0	0	1.2	0	0	0
3-C=O-P	0.2	0.1	-	-	-	-
Carbofuran	15.8	2.7	34.8	18.6	10.8	4.5
3-C=O-CF	2.5	0.5	1.9	1.6	1.3	0.9
3-OH-CF	10.5	2.0	10.0	10.6	5.8	4.8
N-CH_2OH-CF	3.4	1.0	3.0	3.5	2.7	2.8
Unknown	0.2	0.1	0	0	0	0
3-OH-N-CH_2OH-CF	0.3	0.1	0	0	0	0
Unknown II	0.2	0.1	0.3	0.4	0.5	0.7
Sub-total	37.8	7.4	69.3	39.6	24.2	15.3
B. Internal extract						
I. Organo-soluble						
DBSC	1.5	0.7	3.4	2.0	2.4	1.7
Carbofuran	8.2	10.2	14.0	16.1	13.9	7.9
3-C=O-CF	0.7	1.1	0.3	0.4	0.5	0.6
3-OH-CF	}2.2	}10.2	1.0	1.3	1.0	1.4
N-CH_2OH-CF			0.2	0.6	0.7	0.6
Unknown II	0.1	0.2	<0.1	0.1	0.1	0.3
Sub-total	12.7	22.4	18.9	20.5	18.6	12.5
II. Water-soluble conjugate						
Phenol	2.7	2.8	-	-	-	-
3-C=O-P	9.0	9.8	-	-	-	-
3-OH-P	4.5	3.0	-	-	-	-
Carbofuran	0.2	<0.1	0.4	0.2	0.3	0.2
NH_2-CF[b]	4.0	7.4	0.1	0.6	0.6	1.5
3-OH-CF	20.3	31.7	8.6	31.6	44.3	53.9
Unknown I	0.5	0.2	<0.1	0.1	0.1	0.4
3-OH-NH_2-CF[b]	0.8	2.5	<0.1	0.2	0.2	0.7
Unknown II	0.1	3.0	0.2	0.5	0.6	1.1
Unextractable	3.5	4.4	1.7	4.4	7.0	8.6
Sub-total	45.6	64.8	11.0	37.6	53.1	66.4

continued next page

TABLE 7. (cont'd)

Metabolites	Percent of Total DBSC Applied (Days)					
	Ring label		Carbonyl label			
	3	10	1	3	6	10
III. Unextractable (Radioactivity in straw)	4.1	5.4	0.8	2.3	4.1	5.8
Total	100.2	100.0	100.0	100.0	100.0	100.0

[a] Ten microliters of an ethanol-acetone-water (1:3:6) solution containing ring-^{14}C-DBSC (6.6 μg, 0.202 μci) or DBSC-^{14}C-carbonyl (4.98 μg, 0.188 μci) were painted on the two bifoliate leaves of each cotton plant (1.23 g and 1.19 g in fresh weight, respectively) in the early trifoliate stage. There were 3 plants per set.

[b] NH_2-CF and 3-OH-NH_2-CF are artificial products from the acid-hydrolysis treatment and actual metabolites are N-CH_2OH-CF and 3-OH-N-CH_2OH-CF, respectively.

TABLE 8. METABOLISM OF ^{14}C-DIBUTYLAMINOSULFENYL CARBOFURAN (CARBONYL LABEL) IN CORN PLANTS AFTER STEM INJECTION.[a]

Metabolites	Percent of Total DBSC Applied (Days)			
	1	3	7	10
Total recovery	88.1	71.2	49.8	45.6
A. Organo-soluble				
DBSC	23.3	16.8	17.3	7.5
Carbofuran	56.3	39.5	25.1	17.7
3-C=O-CF	1.8	4.3	5.1	5.1
3-OH-CF	9.7	23.4	26.1	27.7
N-CH_2OH-CF	0	0	1.0	2.1
Unknown II	0.2	0.6	1.0	3.4
Sub-total	91.3	84.6	75.6	63.5
B. Water-soluble conjugate				
Carbofuran	1.7	1.0	1.0	0.7
NH_2-CF[b]	0.6	1.0	1.4	1.7
3-OH-CF	0.6	3.6	6.2	11.2
Unknown I	0.1	0.2	0.2	0.6
3-OH-NH_2-CF[b]	0.4	1.2	1.5	3.0
Unknown II	0.5	0.4	0.8	0.8
Unextractable	2.0	2.0	2.2	1.8
Sub-total	5.9	9.4	13.3	19.8
C. Unextractable (Radioactivity in straw)	2.8	6.0	11.1	16.5
Total	100.0	100.0	100.0	99.8

[a] One microliter of an ethanol-acetone (1:1) solution containing carbonyl-^{14}C-DBSC (5.36 μg, 0.202 μci) was injected into the stem of each corn seedling (1.26 g in fresh weight). There were 4 plants per set.

[b] NH_2-CF and 3-OH-NH_2-CF are artificial products from the acid-hydrolysis treatment and actual metabolites are N-CH_2OH-CF and 3-OH-N-CH_2OH-CF, respectively.

between corn and cotton were noted in the amounts of DBSC present in the organo-soluble fraction and the distribution of radioactivity between organo-soluble and water-soluble fractions. In corn, DBSC was converted to carbofuran more rapidly than in cotton and the carbofuran was converted into subsequent metabolites. However, most of these metabolites stayed as unconjugated compounds and formation of conjugates proceeded very slowly. The percentage of radioactivity remaining in the residual straw was higher in corn compared to cotton.

Summary - The metabolic behavior of DBSC and MSC in corn and cotton is summarized by the scheme given (Scheme 1).

The results clearly indicate N-S bond cleavage as the initial step in the metabolic pathway, giving rise to carbofuran as the first major metabolite. Carbofuran then was further metabolized to 3-OH-CF which, in turn, was conjugated. Overall, carbofuran and 3-OH-CF were the major metabolites, with carbofuran being more significant in the early period after treatment and 3-OH-CF becoming more important in the later periods. All other metabolites, including derivatives of the intact carbamate and phenolic derivatives, were of minor significance. No metabolite in which the dibutylaminosulfenyl moiety remained attached to the carbamate linkage was detected. N-S bond cleavage, oxidation, hydrolysis, and conjugation therefore remain the principal reactions in the metabolism of DBSC and MSC in cotton and corn plants.

Qualitatively, the metabolism of DBSC and MSC in corn were similar, but the rates of metabolism of both carbofuran derivatives were slower in corn than in cotton. This is in agreement with previous studies on the metabolism of carbofuran in these crops[3]. Further, conjugation of hydroxylated metabolites took place distinctly slower in corn than in cotton.

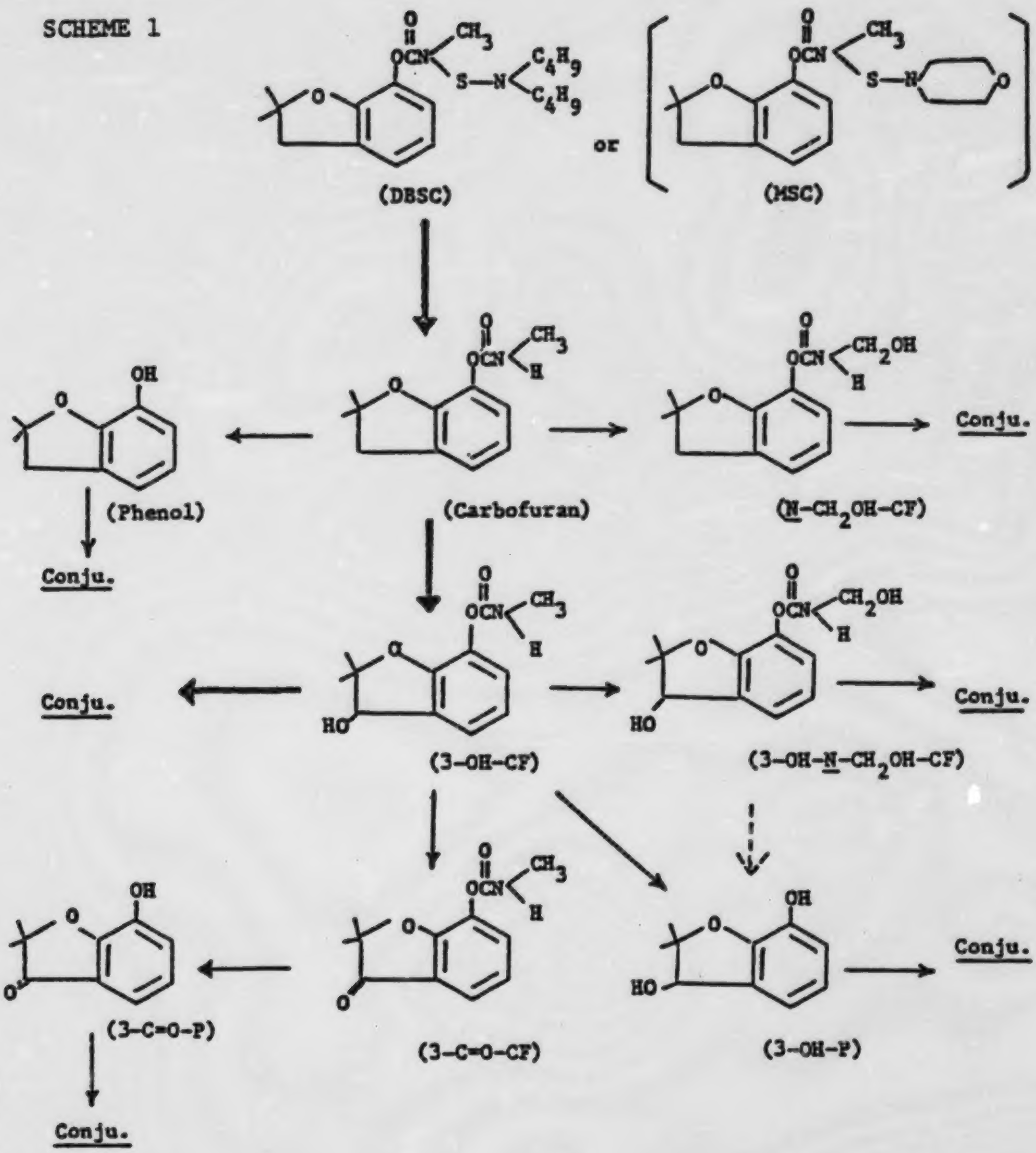
b. Metabolism of DBSC in the Rat.

Introduction - The metabolism of DBSC in the rat was examined to determine the fate of this compound in mammals and to establish the basis for its lower mammalian toxicity relative to carbofuran.

Results - DBSC is metabolized rapidly in the rat. Between 60-90% of the applied radioactivity was excreted in the first 24 hours after oral treatment of ring-labeled, carbonyl-labeled, or butyl-labeled DBSC. There were no significant differences in the metabolism of DBSC in male or female rats, nor was there any significant difference in the metabolism of the two dose levels of DBSC administered (30 mg/kg and 3 mg/kg). The results from the metabolism of DBSC by female rats treated with 30 mg/kg was chosen as representative of all the rats used, and those results are reported here.

The most important means of elimination of orally administered DBSC was via the urine for ring-labeled and dibutyl-labeled DBSC and via expired carbon dioxide and urine for carbonyl-labeled DBSC. Between 70-90% of the applied dose was recovered from the urine of those animals treated with ring-labeled DBSC. Animals treated with dibutyl-labeled DBSC eliminated 50-70% of the

SCHEME 1


(DBSC)
or
(MSC)
(Phenol)
Conju.
(Carbofuran)
(N-CH2OH-CF)
Conju.
Conju.
(3-OH-CF)
(3-OH-N-CH2OH-CF)
Conju.
(3-C=O-P)
Conju.
(3-C=O-CF)
(3-OH-P)
Conju.

applied dose via the urine. Animals treated with carbonyl-labeled DBSC eliminated 30-45% of the applied dose as carbon dioxide and 30-48% of the applied dose via the urine. In the case of all three different labels, elimination of radioactivity via the feces was less important (5-39% of the applied dose). Generally the level of feces elimination was highest from rats treated with dibutyl-labeled DBSC (20-39% of the applied dose). The material balance of excreted radioactivity is presented in Table 9.

Table 10 summarizes the distribution and identity of the metabolites eliminated 24 hours after treatment of a typical female rat with a single dose of ring-labeled DBSC at 30 mg/kg. The values reported are the percentage of the radioactivity recovered after 24 hours (80% of applied dose). Most of the radioactivity was present in the urine as water soluble conjugates. The most important metabolite was 3-ketocarbofuran phenol (36.1% of the 24 hour recovery). 3-Hydroxycarbofuran (15.7%) was the only other metabolite accounting for more than 5% of the recovered dose. Minor metabolites included 3-ketocarbofuran, 3-hydroxycarbofuran phenol, and several N-hydroxymethylcarbofuran species. Good resolution of 3-hydroxy-N-hydroxymethylcarbofuran and 3-keto-N-hydroxymethylcarbofuran by TLC could not be obtained and these values are reported together. A very important metabolite excreted with the carbamate nitrogen-sulfur linkage intact was DBSC-sulfone which was eliminated primarily in the feces. Excreted phenolic metabolites of DBSC accounted for 42.5% of the recovered dose of ring-labeled DBSC; phenolic metabolites were identified exclusively in the urine.

Table 11 summarizes the distribution and identity of the metabolites of carbonyl-labeled DBSC recovered 24 hours after treatment. The values reported are the percent of the radioactivity recovered after 24 hours (70% applied dose). The amount of [^{14}C]carbon dioxide trapped from the expired air of the treated rat was 43.7% of the recovered dose. This value corresponds closely to the amount of phenolic metabolites excreted by animals treated with ring-labeled DBSC (42.5%). The most important metabolite of carbonyl-labeled DBSC was 3-hydroxycarbofuran (22.2%). The identity and the amount of metabolic products excreted by animals treated with carbonyl-labeled DBSC corresponds to the intact carbamate metabolites of ring-labeled DBSC. Metabolites with the N-S bond intact were excreted primarily via the feces. DBSC-sulfone was the most important of these metabolites; smaller amounts of bis-carbofuran disulfide and 3-keto-DBSC were also identified.

Table 12 presents the distribution and identity of the metabolites excreted 24 hours after treatment with dibutyl-labeled DBSC (75% applied dose). The most important metabolite was dibutylamine (53.8% of the 24 hour recovery); it was recovered unconjugated in the urine and trapped as a volatile metabolite. The next most important metabolite was methylaminosulfonyldibutylamine (SO_2-urea) (21.3%) which was recovered in the feces. DBSC-sulfone was also found in the feces.

It was decided to examine the nature of DBSC related compounds in the blood during the intoxication of the rat by DBSC in order to determine the rate of formation of carbofuran from DBSC *in vivo*. The relative amounts of DBSC

TABLE 9. MATERIAL BALANCE FOR FEMALE RATS TREATED AT 30 mg/kg AS A PERCENT OF THE RECOVERED DOSE

	Carbonyl label	Ring label	Butyl label
Urine			
24 hr	27.1	75.9	46.9
48 hr	8.2	6.7	4.2
72 hr	-	1.8	1.7
96 hr	-	1.1	0.6
Feces extractable into acetone-methanol			
24 hr	11.6	4.1	18.7
48 hr	1.6	0.6	2.0
72 hr	-	0.4	0.7
96 hr	-	0.2	5.4
Feces residue after extraction			
24 hr	2.3	1.1	0.6
48 hr	1.1	0.4	0.3
72 hr	-	0.3	<0.1
96 hr	-	0.1	0.3
Cage wash	1.5	1.5	3.5
Tissue	3.9	2.9	0.7
$CO_2/HNBu_2$	38.2	-	14.4
Total recovery of applied dose	98.5	97.2	102.8

TABLE 10. METABOLITES OF A FEMALE RAT TREATED AT 30 mg/kg WITH RING-LABELED DBSC 0.24 HOURS AFTER TREATMENT. VALUES ARE A PERCENT OF THE RECOVERED DOSE

Metabolite	Organic Ext. of urine	Enz. Digest of urine	H^+ Hydrol. of urine	Feces	Total
DBSC-sulfone	0.3			2.7	3.0
CF				1.7	1.7
Phenol		1.0	1.5		2.5
3-C=O-P	1.5	14.0	20.6		36.1
3-C=O-CF	0.4	0.1	0.2		0.7
3-OH-P		1.4	2.5		3.9
3-OH-CF	1.1	6.3	8.3		15.7
NH_2-CF			0.3		0.3
NCH_2OH-CF	0.3	0.7			1.0
3-C=O-NH_2CF			0.2		0.2
3-OH-NH_2CF			0.4		0.4
3-C=O-NCH_2OH-CF +3-OH-NCH_2OH-CF		0.9		0.3	1.2
Origin	0.2	<0.1	0.1	0.3	0.6
Aqueous residue (urine)					31.1
Feces residue					1.4
Total					99.8

TABLE 11. METABOLITES OF A FEMALE RAT TREATED WITH 30 mg/kg OF CARBONYL-LABELED DBSC 0-24 HOURS. VALUES ARE THE PERCENT OF THE RECOVERED DOSE

Metabolite	Organic Ext. of urine	Enz. Digest of urine	H^+ Hydrol. of urine	Feces	Total
DBSC-sulfone	0.6			7.4	8.0
Disulfide	0.3			0.6	0.9
3-C=O-DBSC	0.1				0.1
CF	0.3			1.7	2.0
3-OH-CF	2.6	7.7	11.9	1.6	23.8
NCH_2OH-CF	0.1	0.4			0.5
NH_2-CF			1.1		1.1
3-C=O-NCH_2OH-CF + 3-OH-NCH_2OH-CF	0.4	2.9			3.3
3-C=O-NH_2-CF			1.1		1.1
Origin	0.1	0.3	0.1	0.1	0.6
Aqueous residue (urine)					13.3
Feces residue					1.8
CO_2					43.7
Total					100.0

TABLE 12. METABOLITES OF A FEMALE RAT TREATED WITH 30 mg/kg OF DIBUTYL-LABELED DBSC 0-24 HOURS AFTER TREATMENT. VALUES ARE THE PERCENT OF RECOVERED DOSE

Metabolite	Organic Extract of urine	Basic Extract of urine	Feces	Volatiles	Total
Dibutyl amine	26.9	10.8	0.1	16.0	54.7
DBSC-sulfone			2.4		2.4
SO_2-Urea			21.3		21.3
Unknown	4.9				4.9
Aqueous residue (urine)					16.9
Feces residue					0.8
Total					100.1

and of its metabolites were determined 3 hours after the administration of an oral dose of 30 mg/kg of DBSC. It was found that 73% of the radioactivity recovered from the blood of a rat was unchanged DBSC, 20% was carbofuran, and 7% hydrolysis products. Thus, DBSC was found to be relatively stable in the blood of a rat *in vivo*.

Summary - DBSC is initially metabolized by two major pathways in the rat; via oxidation of the sulfur to give sulfonyl DBSC, and via N-S bond cleavage to give the actual toxicant, carbofuran. Following the initial metabolic degradation of DBSC to carbofuran, carbofuran was detoxified primarily via oxidation at the 3-carbon position of the dihydrofuranyl ring. The primary excreted metabolite was 3-ketocarbofuran phenol, followed by 3-hydroxycarbofuran. Minor metabolites included carbofuran phenol, 3-hydroxycarbofuran phenol, 3-ketocarbofuran, and species oxidized at the *N*-methyl position.

While DBSC is converted to carbofuran in the rat, it appears that the rate of formation of carbofuran is slower than expected from studies on its degradation under different pH conditions. Based on the high levels of DBSC present in the blood 3 hours after treatment, it appears that carbofuran is released slowly and this slow release, in part, accounts for the lower mammalian toxicity of DBSC.

c. Metabolism in Soil.

Introduction - The preceding section described the metabolic fate of MSC and DBSC in corn and cotton plants. In ongoing studies, the fate and metabolism of these two carbofuran derivatives also were examined in soil. This section is concerned with the alteration of MSC and DBSC in Cosad sandy loam soil under aerobic and anaerobic conditions. The degradation of the major primary metabolites, carbofuran and dibutylamine, also was investigated.

Identification of Compounds - Structures and number designations of compounds used in this study are given in Table 13.

Aerobic Degradation of DBSC - Data giving the distribution and nature of radioactive products recovered from soil after incorporation of 30 ppm [carbonyl-^{14}C]DBSC are presented in Table 14. DBSC, with a half-life of about 3 days, was rapidly converted to carbofuran which remained as the major radioactive component during the 30-day test period. The other prominent radioactive component was carbon dioxide, particularly during the later stages of the experiment. Radioactivity bound to the soil following water-methanol and dichloromethane extractions, was distinctly higher in the early stages of the incubation period, suggesting that the initial residue became more accessible to extraction and/or more available to degradation. Compounds III and XI were observed in minor quantities.

TABLE 13. STRUCTURES OF DBSC AND AUTHENTIC STANDARDS

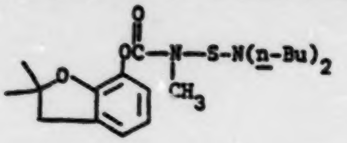
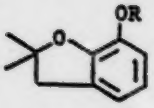
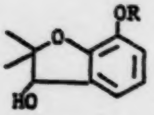
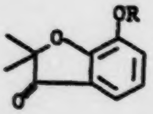
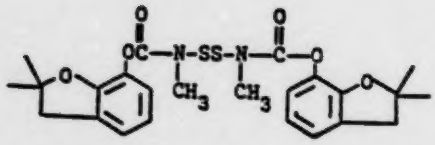
	$\underline{R}$	Designation
		DBSC
	$C(O)NHCH_3$	I (Carbofuran)
	$C(O)NHCH_2OH$	II
	$C(O)NH_2$	III
	H	IV
	$C(O)NHCH_3$	V
	$C(O)NHCH_2OH$	VI
	$C(O)NH_2$	VII
	H	VIII
	$C(O)NHCH_3$	IX
	$C(O)NHCH_2OH$	X
	$C(O)NH_2$	XI
	H	XII
		XIII
$(\underline{n}\text{-Bu})_2NSN(\underline{n}\text{-Bu})_2$		XIV
$(\underline{n}\text{-Bu})_2NSSN(\underline{n}\text{-Bu})_2$		XV
$(\underline{n}\text{-Bu})_2NSSSN(\underline{n}\text{-Bu})_2$		XVI
$HN(\underline{n}\text{-Bu})_2$		XVII (Dibutylamine)
$CH_3(CH_2)_2COOH$		XVIII
$CH_3NH\text{-}SO_2\text{-}N(\underline{n}\text{-Bu})_2$		XIX

TABLE 14. PERCENTAGE OF APPLIED RADIOACTIVITY RECOVERED AS DBSC AND DEGRADATION PRODUCTS FROM AEROBIC COSAD SANDY LOAM AFTER TREATMENT WITH 30 PPM [CARBONYL-^{14}C]DBSC.

	Incubation Time (days)						
	0	2	5	10	16	23	30
Carbon dioxide	-	0.2	1.5	6.5	12.3	22.9	26.8
Aqueous extract	0.1	0.3	0.4	0.6	0.7	0.7	0.6
Dichloromethane extract	81.0	69.2	76.6	77.5	75.5	63.3	53.6
DBSC	79.9	46.1	33.5	20.8	11.8	5.5	3.4
I (carbofuran)	1.0	23.0	42.1	54.6	61.2	55.9	47.9
III	-	-	0.1	0.5	0.6	0.4	0.4
IV	-	-	0.7	1.6	2.0	1.4	1.9
Origin	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Initial residue	24.5	26.1	20.8	16.7	16.4	15.6	14.6
Total recovered	105.6	95.8	99.3	101.3	104.9	102.5	95.7

Similar experiments were carried out with [carbonyl-^{14}C]MSC (morpholinosulfenyl carbofuran) in aerobic Cosad sandy loam.

Similar data for [ring-^{14}C]DBSC breakdown are given in Table 15. The results were in general agreement with data obtained with the [carbonyl-^{14}C]-material and carbofuran, again, was the major degradation product. In addition to III and IX, other minor components included IV, XII, and one unknown. A small but increasing amount of [^{14}C]carbon dioxide was trapped, providing an indication of the slow degradation of the aromatic ring.

Based on the amount of [^{14}C]carbon dioxide evolved from [carbonyl-^{14}C]DBSC (27% after 30 days), the amount of [ring-^{14}C]phenolic products (IV, VIII, and XII) was expected to be larger, i.e., roughly equal to the amounts of carbon dioxide produced from the [carbonyl-^{14}C]DBSC. However, relatively small amounts of [^{14}C]phenolic products were recovered, and, in contrast to results from the [carbonyl-^{14}C]DBSC, a substantial increase in the amount of radioactivity in the initial residue was noted. These results indicate the binding of IV or its oxidation products (VIII and XII) to soil matter, probably to soil humus[4] and concomitant decrease in the amount of phenols available for immediate decomposition. Thus, the slow rate of evolution of [^{14}C]carbon dioxide from [ring-^{14}C]DBSC may be attributable not only to the stability of the aromatic ring but also to the incorporation of the ring to soil organic matter.

TABLE 15. PERCENTAGE OF APPLIED RADIOACTIVITY RECOVERED AS DBSC AND DEGRADATION PRODUCTS FROM AEROBIC COSAD SANDY LOAM AFTER TREATMENT WITH 30 PPM [RING-^{14}C]DBSC.

	INCUBATION TIME (days)							
	0	2	5	10	16	23	30	60
Carbon dioxide	-	0.0	0.3	1.7	4.3	6.9	8.8	13.1
Aqueous extract	0.1	0.2	0.3	0.5	0.7	0.9	1.0	0.9
Dichloromethane extract	75.9	81.7	81.3	81.1	70.1	60.3	53.5	35.9
DBSC	75.0	63.5	45.0	25.9	15.9	8.5	4.7	1.7
I (carbofuran)	0.8	18.0	35.3	52.6	50.9	47.5	46.3	33.0
III	-	0.1	0.1	0.4	0.3	0.4	0.3	0.6
IV	-	-	-	-	0.4	0.3	0.2	0.3
IX	-	0.2	0.3	0.7	0.8	1.1	1.0	-
XII	-	-	0.2	1.1	1.1	1.8	0.4	-
Unknown	-	-	-	0.4	0.7	0.8	0.6	0.1
Origin	0.2	0.3	0.3	0.2	0.2	0.1	0.1	0.3
Initial residue[a]	26.3	22.1	21.4	23.2	26.8	32.1	34.4	37.9
Total recovered	102.3	103.9	103.3	106.5	101.9	100.2	97.6	87.8

[a] Initial residue is the sum of the benzene, acetone and crude hydrolysis extracts, and bound residue

Data obtained from the aerobic degradation of [ring-^{14}C]DBSC incubated in soil at 3 ppm were similar to those obtained at 30 ppm. At both concentrations the breakdown of DBSC was first-order.

In an effort to characterize the radioactivity in the initial residue remaining after primary extraction, the soil was exhaustively extracted with benzene and acetone. The results of the benzene and acetone extractions and acid hydrolysis of the soil treated with [ring-^{14}C]DBSC are shown in Table 16. Since DBSC easily decomposes under acidic conditions, no DBSC was expected in the dichloromethane hydrolysis extract. The data show that the amount of radioactivity in the initial residue increased with time along with a decrease in the amount of radioactivity extracted by benzene and acetone. This indicates that most of the radioactivity in the initial residue was available for extraction (loosely bound) and became less available with time. The amount of radioactivity extracted in the crude hydrolysis extract remained constant, but the amount of radioactivity lost in cleanup of the extract increased with time. The cleanup procedures removed large amounts of biological material which were solubilized by the acid hydrolysis procedures. Thus, the increase in the radioactivity lost in cleanup indicates a buildup of radioactivity in the soil organic matter. The bound residue which contained the unsolubilized organic matter also showed a steady increase in radioactivity with time. These data also support the conclusion that the nature of the binding of the radioactive products to soil changed with time.

TLC analysis of the secondary organic solvent extracts (benzene extract, acetone extract, and dichloromethane hydrolysis extract) revealed the composition of a portion of the loosely bound radioactivity in the initial residue. At time zero, approximately 86% of the radioactivity remaining after the primary extractions was carbofuran; however, after 30 days incubation, the level of carbofuran decreased to 23% of the initial residue. In addition to the degradation products shown in Table 16, small amounts (less than or equal to 0.1% of the applied radioactivity) of the following degradation products were detected: benzene extract contained III, IV, V, VI, IX, and XII; acetone extract contained III; and dichloromethane hydrolysis extract contained III, IV, IX, and XII.

The data showing distribution of the [^{14}C]degradation products of [dibutylamino-^{14}C]DBSC in soil is given in Table 17, column a. The maximum amount of XVII was 5.3% while, after 30 days, 51.3% and 28.5% of the applied radioactivity was detected as [^{14}C]carbon dioxide and as the initial residue, respectively. These data suggest that the dibutylamine containing degradation products of DBSC are quickly decomposed or bound to the soil. Less than 1% of the applied radioactivity was recovered in the 30-day benzene and acetone extracts. A large fraction of the radioactivity recovered in the crude hydrolysis extract was lost during the cleanup procedure. Since the cleanup procedure removes most of the acid solubilized organic matter, it appears that most of the radioactivity extracted after the hydrolysis was actually bound to the acid soluble soil organic matter.

TABLE 16. PERCENTAGE OF APPLIED RADIOACTIVITY RECOVERED AS DBSC AND DEGRADATION PRODUCTS FROM AEROBIC COSAD SANDY LOAM AFTER TREATMENT WITH 30 PPM [RING-^{14}C]DBSC.

	INCUBATION TIME (Days)							
	0	2	5	10	16	23	30	60
Initial residue[a]	26.3	22.1	21.4	23.2	26.8	32.1	34.4	37.9
Benzene extract	10.0	8.1	6.3	4.1	3.3	2.8	1.9	0.5
DBSC	2.9	2.5	2.6	1.0	1.0	0.7	0.3	0.1
I (carbofuran)	6.8	5.4	3.6	3.0	2.2	2.0	1.6	0.4
Unknown	0.1	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	-
Origin	<0.1	<0.1	0.0	<0.1	0.0	0.0	0.0	0.0
Acetone extract	6.8	2.0	5.2	2.4	2.7	2.2	1.3	0.8
DBSC	0.3	<0.1	0.2	0.1	0.1	0.1	0.1	0.1
I	6.1	1.8	4.8	2.2	2.5	2.0	1.2	0.7
Unknown	0.2	<0.1	0.1	0.2	0.1	0.1	0.1	-
Origin	0.2	<0.1	0.1	<0.1	0.1	0.1	0.1	0.0
Crude hydrolysis extract	8.5	10.0	7.0	9.0	9.1	10.0	11.9	10.8[b]
Aqueous hydrolysis extract	0.1	0.1	0.1	0.2	0.4	0.4	0.7	1.2
Dichloromethane hydrolysis extract	6.3	7.8	5.2	6.1	4.9	4.8	5.1	4.0
I	6.2	7.7	5.1	5.9	4.8	4.6	4.8	3.6
Origin	<0.1	<0.1	<0.1	<0.1	<0.1	0.1	0.1	0.1
Bound residue	1.0	2.0	2.9	7.7	11.7	17.1	19.3	25.8[b]

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[a] Initial residue is the sum of the benzene, acetone, and crude hydrolysis extracts, and bound residue.

[b] Values determined from 1 incubation flask.

TABLE 17. PERCENTAGE OF APPLIED RADIOACTIVITY RECOVERED AS DBSC OR DIBUTYLAMINE AND THEIR DEGRADATION PRODUCTS FROM AEROBIC COSAD SANDY LOAM AFTER TREATMENT WITH 30 PPM [DIBUTYL-AMINO-^{14}C]DBSC OR [^{14}C]DIBUTYLAMINE.

INCUBATION TIME (days)	a. DBSC				b. Dibutylamine
	0	2	16	30[a]	30
Carbon dioxide	-	2.4	23.8	51.3	51.1
Aqueous extract	0.6	1.5	1.1	1.2	0.8
Dichloromethane extract	75.1	66.7	37.0	10.4	0.7
DBSC	69.8	58.9	36.1	9.8	-
XVII	2.9	5.3	0.4	0.1	0.3
XIX	0.2	0.1	-	-	-
Unknowns (6)[b]	0.3	0.2	0.1	0.2	0.2
Origin	1.9	2.2	0.4	0.2	0.2
Initial residue	23.1	28.2	29.8	29.9[c]	20.8[c]
Benzene extract	-	-	-	0.4	0.1
Acetone extract	-	-	-	0.1	0.3
Crude hydrolysis extract	-	-	-	7.6	6.6
Aqueous hydrolysis extract	-	-	-	0.2	0.3
Dichloromethane hydrolysis extract	-	-	-	0.8	1.3
Bound residue	-	-	-	21.8	13.8
Total recovered	98.9	98.7	91.8	92.8	73.5

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[a] Values are the mean of 4 replications.

[b] Number of unknown compounds represented is shown in parentheses.

[c] Values are the sum of the benzene, acetone, and crude hydrolysis extracts and bound residue.

The fate of [^{14}C]dibutylamine, incubated in aerobic soil for 30 days, is shown in Table 18, column b. Overall, the results are similar to that obtained after the incubation of [dibutylamino-^{14}C]DBSC. Less than 1% of the applied radioactivity was recovered in the dichloromethane extract of the dibutylamine fortified soil, virtually identical to results obtained after incubation of [dibutylamino-^{14}C]DBSC.

Anaerobic Degradation of DBSC - The results of the anaerobic decomposition of [carbonyl-^{14}C]DBSC are shown in Table 18. As in the case of the aerobic studies, DBSC rapidly decomposed to form carbofuran. Compared to the aerobic study, larger amounts of carbofuran were observed in the dichloromethane extract and it appeared to be more stable under anaerobic conditions. The amount of radioactivity remaining on the soil decreased with time, indicating that the initial residue was loosely bound and/or susceptible to degradation. In contrast to the aerobic decomposition of DBSC, III and IX were not detected. Since these degradation products arise from oxidative processes, they would not be expected under anaerobic conditions.

Table 19 contains data obtained from the primary extraction of the soil after an anaerobic incubation of [ring-^{14}C]DBSC. Very little [^{14}C]carbon dioxide was recovered, indicating that the ring was not cleaved under anaerobic conditions. The presence of IV as a major degradation product in the dichloromethane extract indicates that phenolic binding mechanisms do not operate in the absence of molecular oxygen. In anaerobic soil, the breakdown of DBSC was first-order with a half-life of about 2 days.

The data in Table 20 were obtained after secondary extraction with benzene and acetone and acid hydrolysis of the anaerobic soil treated with [ring-^{14}]DBSC. TLC analysis of the extracts showed that the radioactive components of initial residue were composed mostly of intact carbamates. In contrast to the aerobic study (see Table 16), the amount of radioactivity in the bound residue did not increase after day 5 under anaerobic conditions. This suggests that the radioactivity in the bound residue of the aerobic soil was bound by a mechanism that requires oxygen.

The results of the anaerobic incubation of [dibutylamino-^{14}C]DBSC are shown in Table 21. In contrast to previous anaerobic incubations, large amounts (9.9 to 38.2%) of the applied radioactivity were detected in the aqueous and aqueous hydrolysis extracts. TLC analysis of the alkaline and alkaline hydrolysis extracts reveals that 15-54% of the radioactivity in the aqueous extract and aqueous hydrolysis extract was XVII.

Summary - DBSC or 2,3-dihydro-2,2-dimethyl-7-benzofuranyl (di-n-butylaminosulfenyl)(methyl)carbamate] was rapidly degraded in Cosad sandy loam with a half-life of about 2-3 days. Thiolysis of DBSC was first-order. DBSC degraded to carbofuran, which was either oxidized at the 3 position of the ring, or hydrolyzed at the carbamate ester to form carbofuran phenol. Biscarbofuran-disulfide, dibutylamine, and at least 7 unidentified minor compounds were also detected. Phenolic degradation products appeared to

TABLE 18. PERCENTAGE OF APPLIED RADIOACTIVITY RECOVERED AS DBSC AND DEGRADATION PRODUCTS FROM ANAEROBIC COSAD SANDY LOAM AFTER TREATMENT WITH 30 PPM [CARBONYL-^{14}C]DBSC

	INCUBATION TIME (days)						
	0	2	5	10	16	23	30
Carbon dioxide	-	0.0	0.1	0.2	0.8	1.2	2.4
Aqueous extract	0.3	0.4	0.6	1.4	0.8	1.4	1.3
Dichloromethane extract	89.6	88.4	92.3	93.7	94.5	93.3	89.4
DBSC	86.6	66.5	24.6	7.6	2.8	1.4	1.4
I	2.8	21.7	67.5	86.0	91.6	91.8	87.9
Origin	0.2	0.2	0.2	0.1	0.1	0.1	0.1
Initial residue	18.3	17.4	15.0	10.5	9.3	10.6	9.6
Total recovered	108.2	106.2	108.0	105.8	105.4	106.5	102.7

TABLE 19. PERCENTAGE OF APPLIED RADIOACTIVITY RECOVERED AS DBSC AND DEGRADATION PRODUCTS FROM ANAEROBIC COSAD SANDY LOAM AFTER TREATMENT WITH 30 PPM [RING-^{14}C]DBSC

	INCUBATION TIME (days)						
	0	2	5	10	16	23	30
Carbon dioxide	-	0.0	0.0	0.0	0.1	0.4	0.9
Aqueous extract	0.1	0.3	0.2	0.3	0.4	0.2	0.3
Dichloromethane extract	87.0	83.4	87.5	90.6	90.1	91.5	93.9
DBSC	80.4	67.7	31.8	12.0	4.2	2.0	2.7
I	0.8	15.0	53.8	76.3	82.9	83.4	80.6
IV	0.5	0.3	0.8	1.4	2.1	4.9	9.3
Unknown	<0.1	0.2	0.8	0.7	0.9	1.1	1.0
Origin	0.2	0.3	0.2	0.3	0.1	0.1	0.2
Initial residue[a]	17.7	17.1	13.3	10.5	10.1	9.0	8.4
Total recovered	99.8	100.8	101.0	101.4	100.7	101.1	103.5

[a] Initial residue is the sum of the benzene, acetone, and crude hydrolysis extracts and the bound residue

TABLE 20. PERCENTAGE OF APPLIED RADIOACTIVITY RECOVERED AS DBSC AND DEGRADATION PRODUCTS FROM ANAEROBIC COSAD SANDY LOAM AFTER TREATMENT WITH 30 PPM [RING-^{14}C]DBSC.

	INCUBATION TIME (days)						
	0	2	5	10	16	23	30
Initial residue[a]	17.7	17.1	13.3	10.5	10.1	9.0	8.4
Benzene extract	2.4	2.7	1.1	0.9	0.3	0.3	0.3
DBSC	0.6	1.0	0.2	0.2	0.1	0.1	0.1
I	1.7	1.7	0.9	0.8	0.3	0.3	0.3
Origin	0.2	0.0	0.0	0.0	0.0	0.0	0.0
Acetone extract	8.2	5.5	2.7	1.3	1.4	0.8	0.4
DBSC	1.7	1.1	0.2	0.1	0.1	0.1	0.1
I	6.2	4.2	2.4	1.3	1.3	0.8	0.6
XIII	0.2	0.1	0.1	-	-	-	-
Origin	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Crude hydrolysis extract	6.5	6.9	5.4	4.8	4.5	4.6	4.4
Aqueous hydrolysis extract	0.3	0.1	0.1	0.1	0.1	0.1	0.1
Dichloromethane hydrolysis extract	6.2	6.3	4.7	4.0	4.5	3.8	3.5
I	6.1	6.3	4.6	3.9	4.3	3.7	3.4
III	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Origin	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Bound residue	0.6	2.0	4.1	3.5	3.9	3.3	3.3

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[a] Initial residue is the sum of the benzene, acetone, and crude hydrolysis extracts and the bound residue.

TABLE 21. PERCENTAGE OF APPLIED RADIOACTIVITY RECOVERED AS DBSC AND DEGRADATION PRODUCTS FROM ANAEROBIC COSAD SANDY LOAM AFTER TREATMENT WITH 30 PPM [DIBUTYLAMINO-^{14}C]DBSC.

	INCUBATION TIME (days)		
	2	16	30
Carbon dioxide	0.4	2.0	3.8
Aqueous extract	9.9	19.4	15.5
Alkaline extract	6.7	12.2	12.7
XVII	5.4	7.0	8.3
Unknowns (7)[a]	1.3	2.7	3.7
Origin	0.1	2.5	0.7
Dichloromethane extract	58.1	32.3	21.4
DBSC	43.1	6.2	1.4
XVII	11.7	21.1	16.8
Unknowns (6)[a]	2.9	3.1	2.1
Origin	0.4	1.9	1.1
Initial residue[b]	32.2	44.1	55.9
Benzene extract	0.7	0.3	0.2
Acetone extract	1.6	0.5	0.4
Crude hydrolysis extract	27.0	37.8	47.6
Dichloromethane hydrolysis extract	1.6	1.5	1.5
Aqueous hydrolysis extract	20.6	31.3	38.2
Alkaline hydrolysis extract	8.5	5.8	10.6
XVII	7.7	5.0	9.1
Unknowns (6)[a]	0.8	0.6	1.5
Origin	<0.1	0.2	<0.1
Bound residue	2.9	5.5	7.7
Total recovered	100.6	97.8	96.6

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[a] Number of unknown compounds represented is shown within the parenthesis.

[b] Initial residue is the sum of the benzene, acetone, and crude hydrolysis extracts and bound residues.

be bound to the soil humus by an oxygen-dependent process. Also, ring cleavage was found to be an oxygen-dependent process.

d. Alteration of DBSC in a Water Environment.

Introduction - Ongoing studies on the behavior of DBSC in different environments, e.g., soil and sand, raised the question of the importance of nonbiological alteration in the breakdown of DBSC. A study, therefore, was undertaken to determine the reactions involved in the degradation of DBSC and the resulting breakdown products under different environmental conditions. This section is concerned with the breakdown of DBSC in different buffer solutions, in buffer solutions containing sulfhydryl reagents, and on the surface of silica gel TLC plates.

Alteration of DBSC in Different Buffer Solutions - DBSC was unstable in acidic buffer (pH 3.0 and 5.0), rapidly decomposing to carbofuran as the principal product. Several other products of relatively minor significance also were observed. Compounds which were identified were biscarbofuran disulfide (Disulfide) and DBSC-sulfone. Three minor unknowns also were detected.

Quantitative data showing the relative amounts of the various alteration products formed after allowing [carbonyl-^{14}C]DBSC to stand in methanol-buffer media at pH 3.0, 5.0, 7.0 and 9.0 are presented in Table 22. At pH 3.0, 93% of the DBSC introduced into the buffer was converted to carbofuran after 10 min. The rate of conversion of DBSC into carbofuran decreased with increasing pH and about 33% of the initial DBSC was recovered as carbofuran after 1.5 hr in pH 5.0 buffer. None of the other alteration products was found in quantity greater than 2% of the total radioactivity. DBSC was substantially more stable in neutral buffer (pH 7.0) where only 15% of the total radioactivity was recovered in the form of carbofuran after 48 hr. In contrast to results in buffers of lower pH a significant amount (12.3%) of an unknown product (Unknown III) was isolated from the neutral buffer. DBSC was even more stable at pH 9.0 than at neutral pH, returning 86% of the total radioactivity as DBSC after 48 hr.

The degradation of DBSC in pH 5.0, 7.0 and 9.0 buffer was first-order.

Alteration of DBSC in Buffer Containing Sulfhydryl Agents - The effect of L-cysteine and reduced glutathione on the breakdown of DBSC in the different buffers was examined with [carbonyl-^{14}C]DBSC under a nitrogen atmosphere. DBSC was allowed to stand in pH 5.0 and 7.0 buffer containing a 26-mole excess of cysteine or glutathione. At pH 7.0, both cysteine and reduced glutathione rapidly effected the breakdown of DBSC, resulting in carbofuran as the sole radiolabeled degradation product. The rate of DBSC conversion to carbofuran in the presence of excess cysteine or reduced glutathione was first order. The calculated first-order constants showed that the decomposition of DBSC in the presence of cysteine and reduced glutathione was 348- and 174-fold faster, respectively, than in buffer alone. It is apparent that reagents containing a sulfhydryl moiety rapidly react with DBSC to cleave the carbamate nitrogen-sulfur bond and liberate carbofuran.

TABLE 22. DEGRADATION OF CARBONYL-^{14}C-DBSC IN DIFFERENT METHANOL (40%)-BUFFER MIXTURE[a]

Compound	Percent of Total DBSC Applied						
	Control (pH 9.0, 10 min)	pH 3.0	pH 5.0		pH 7.0		pH 9.0
		10 min	1.5 hr	5 hr	6 hr	48 hr	48 hr
Unknown I	-	0.6	-	-	-	-	-
DBSC	95.9	2.0	62.6	30.0	}92.7	}68.9	}86.1
DBSC-sulfone	-	0.8	1.8	1.4			
Disulfide	0.9	1.5	0.8	1.1	1.1	2.9	1.3
Unknown II	0.8	2.0	1.1	0.9	0.6	0.5	
Carbofuran	2.0	92.7	32.7	64.9	3.9	15.3	6.2
Unknown III (original spot)	0.5	1.9	0.2	1.6	1.4	12.3	6.0
Total	100.1	100.1	100.1	100.1	100.0	100.0	100.1

[a] One µl of ethanol containing [carbonyl-^{14}C]-DBSC (0.44 µCi, 0.031 µmole) was added into 199 µl of methanol(40%)-buffer mixture at pH 3.0 and 5.0 (citrate-phosphate buffer, 0.05 M), 7.0 (phosphate buffer, 0.05 M) and 9.0 (boric acid-borax buffer, 0.05 M), and kept at 26°C with gentle shaking.

Alteration of DBSC on TLC Plates - The decomposition of DBSC on silica gel TLC plates upon standing in air or nitrogen was investigated with both [carbonyl-^{14}C]DBSC and [ring-^{14}C]DBSC. DBSC was unstable on silica gel plates and cleavage of each of the N-S bonds occurred, resulting in carbofuran as the major product along with minor amounts of biscarbofuran-*N*,*N*'-disulfide (Disulfide) and the corresponding Trisulfide. Carbonyl and ring labeled material gave the same products. Carbofuran and the Disulfide were identified by cochromatography, NMR and mass spectroscopy while the Trisulfide was identified by NMR and mass spectroscopy.

Significant amounts (up to 25%) of the unknown III, identical to the unknown isolated when DBSC was allowed to stand in buffer, was obtained. The structure of this compound remains unknown. Figure 1 provides plots showing the relative amounts of products formed from DBSC on TLC plates at different time intervals. The gradual disappearance of DBSC and concomitant formation of carbofuran and unknown III as major products and Disulfide and Trisulfide as minor products are clearly indicated.

Summary - The cleavage of the N-S bond in 2,3-dihydro-2,2-dimethyl-7-benzofuranyl (di-*n*-butylaminosulfenyl)(methyl)carbamate was examined in different buffer solutions (hydrolysis), in buffer solution containing sulfhydryl reagents (thiolysis) and on thin-layer chromatographic plates. In buffer solution and on thin-layer plates, N-S bond cleavage readily occurred to give carbofuran as a major product, with minor amounts of bis-carbofuran-*N*,*N*'-disulfide and -trisulfide. The hydrolysis reaction in buffer proceeded with first-order kinetics. Significant amounts of an unknown polar compound were obtained in buffer solution and on thin-layer plates. In the presence of excess cysteine and glutathione at pH 7.0, thiolytic N-S bond cleavage occurred with first-order kinetics to give carbofuran as the sole identifiable product. At pH 5.0, three minor products were obtained along with carbofuran.

e. Acid-Catalyzed Alteration of DBSC.

Introduction - The previous section described the breakdown of DBSC in aqueous buffers of different pH. During the course of a study on the behavior of DBSC under different solvent conditions, we discovered that DBSC also was unstable in a dichloromethane-acetic acid (9:1) mixture, being converted into a variety of different products in significant quantities, in addition to carbofuran. Because of the possible significance of acid catalyzed non-enzymatic alteration on the mode of action and toxicological properties of DBSC, a further probe into the nature of the alteration products was made. Initial studies were made in an aprotic, enert solvent in order to identify all alteration products that were formed from DBSC in an acidic environment.

DBSC Stability in Dichloromethane Containing Acetic Acid or Triethylamine - Preliminary experiments were carried out to determine the stability of DBSC in dichloromethane in the presence of an acid (acetic) and organic base (triethylamine). In the dichloromethane-triethylamine mixture (9:1), and also in dichloromethane alone, DBSC was stable and most of the radio-

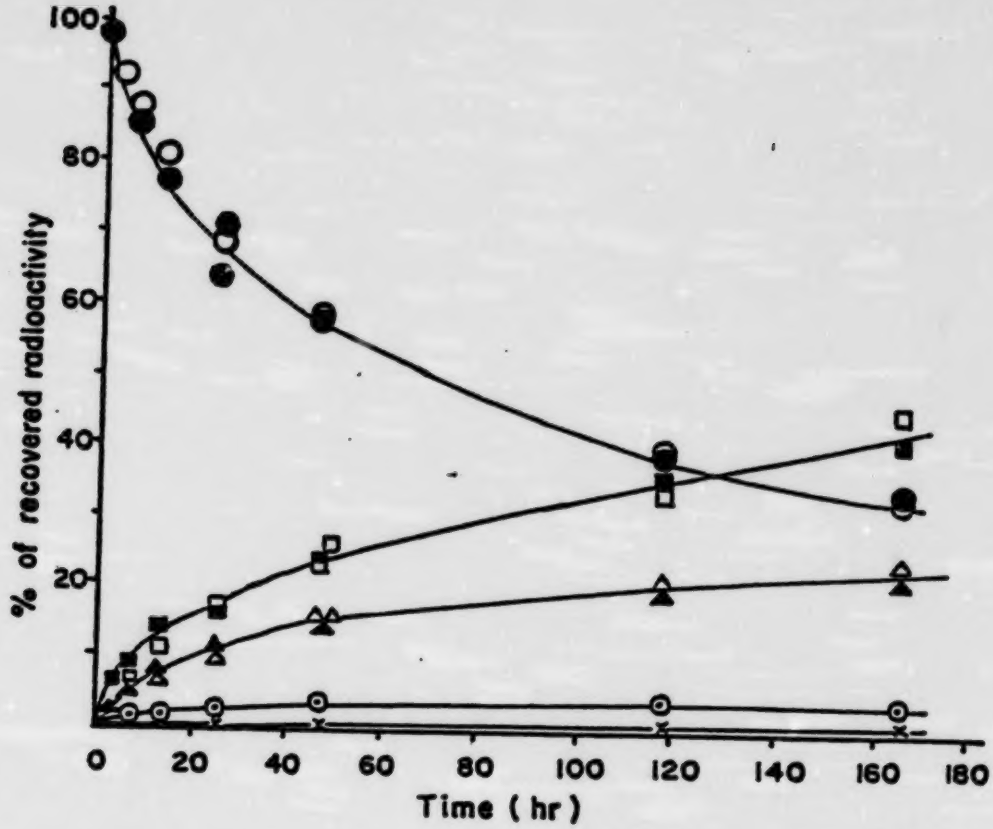


FIGURE 1

Time course of the degradation of [carbonyl-^{14}C]DBSC and [ring-^{14}C]-DBSC on silica gel TLC plate. (●) DBSC (carbonyl label); (■) carbofuran (carbonyl label); (▲) unknown III (carbonyl label); (⊙) Disulfide (carbonyl label); (×) unknown II (a mixture of Trisulfide and an unknown compound, carbonyl label); (○) DBSC (ring label); (□) carbofuran (ring label); (△) unknown III (ring label); Ring labeled Disulfide and unknown II were omitted from the figure.

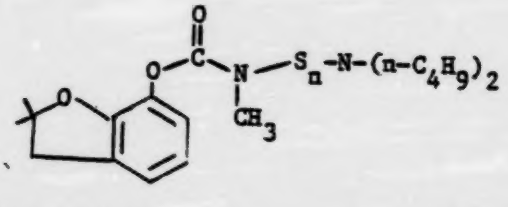
activity was recovered as unchanged DBSC, although trace amounts of several alteration products were detected. For example, after 48 hours, 93.0% of the charged radioactivity was recovered as DBSC from dichloromethane and 93.1% was recovered from the dichloromethane-triethylamine mixture. Recovery of DBSC was virtually the same from the same two solvents after 96 hours.

In contrast, DBSC was highly unstable in dichloromethane-acetic acid, being converted into at least five other materials within 24 hours (see Table 23). TLC analysis (cochromatography with available standards) showed that two of the products were biscarbofuran-N,N'-disulfide (III or CF-S_2-CF) and carbofuran (V). After 24 hours, only 34% of the radioactivity was returned as DBSC, 32% as carbofuran, and 34% as other products (I, III, IV and VI). Recovery of DBSC was 10.7% after 48 hours and 1.3% after 96 hours. Thus, after 96 hours in dichloromethane-acetic acid DBSC was almost completely transformed into other compounds.

Isolation and Identification of DBSC Alteration Products Formed in Dichloromethane-Acetic Acid - Three separate experiments were carried out to isolate and characterize the different alteration products formed in the presence of acetic acid. The following is typical.

Experiment 1. DBSC (1.6 g) in dichloromethane-acetic acid solvent was stirred for 96 hours at 23°C. TLC analysis of the products using hexanes-ether (7:3) as the developing solvent showed the presence of 6 different products (I-VI). The individual products were separated by preparative TLC (20 plates) according to the following R_f values: 169 mg I, R_f 0.50-0.60 (light yellow gum); 155 mg II, R_f 0.41-0.5 (DBSC); 107 mg III, R_f 0.24-0.29 (white solid); 114 mg IV, R_f 0.18-0.24 (white solid); 393 mg V and VI, R_f 0-0.14 (light yellow solid).

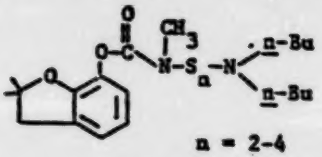
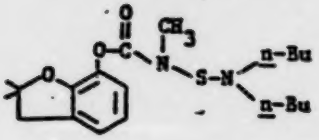
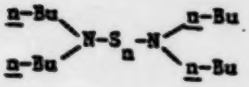
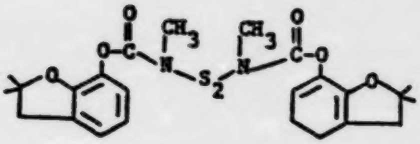
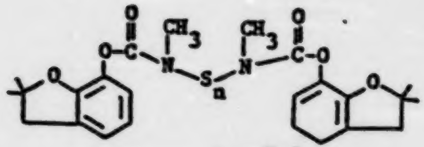
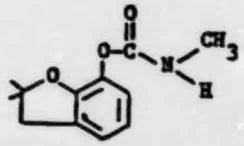
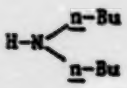
I (89 mg) was further purified by TLC using hexanes-ether (3:1) as the developing solvent. I contained a small amount of I-a which was separated by TLC. The NMR was very similar to that of DBSC except for the upfield shift of the N-CH_2 signals and the appearance of the N-CH_3 protons as a multiplet instead of as a singlet in DBSC. The mass spectrum of I showed three different molecular ion peaks at m/3 412 (CF-S_2-NBu_2), 444 (CF-S_3-NBu_2), and 476 (CF-S_4-NBu_2) along with peaks at 285 (CF-S-SH) and 192 (S=S-NBu_2). These results, combined with the multiplet observed for the NMR signals for the N-CH_3 protons, suggest I to have the following structure:



n = 2-4

Elemental analysis of I gave C, 55.60%; H, 6.84%; S, 20.9%, indicating that the average value for n is close to 3. The percentage of sulfur in CF-S_3-NBu_2 is 21.6.

TABLE 23. STRUCTURE AND TLC PROPERTIES OF DBSC AND ITS ALTERATION PRODUCTS.

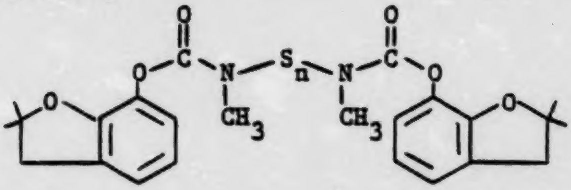
			Solvent[a]	
No.	Abbreviated Name	Structure	A	B
I	$CF-S_n-NBu_2$	 n = 2-4	0.49	0.91
II	DBSC		0.43	-
I-a	$Bu_2N-S_n-NBu_2$	 n = 1-4	0.71	-
III	$CF-S_2-CF$		0.26	-
IV	$CF-S_n-CF$	 n = 3-5	0.23	-
V	Carbofuran		0.10	0.90
VII	Dibutylamine		0	0.52

[a] Solvent system: (A) hexanes-ether (7:3); (B) ethyl acetate-methanol-ammonium hydroxide (15:4:1).

I-a cochromatographed with authentic samples of bis(di-n-butylamino)-N,N'-disulfide ($Bu_2N-S_2-NBu_2$) and bis(di-n-butylamino)-N,N'-trisulfide ($Bu_2N-S_3-NBu_2$), these two compounds having the exact same R_f values on silica gel plates with a variety of different solvent systems. The mass spectrum of I-a showed at least four different molecular ion peaks at m/e 288 ($Bu_2N-S-NBu_2$), 320 ($Bu_2N-S_2-NBu_2$), 352 ($Bu_2N-S_3-NBu_2$) and 384 ($Bu_2N-S_4-NBu_2$). I-a is probably $Bu_2N-S_n-NBu_2$ where n is 1 to 4.

III (54 mg) was subjected to further purification by TLC, using two migrations of hexanes-ether (7:3) and 38 mg of a white crystalline solid, mp 133-134°C, were obtained. NMR and mass spectral data reveal III to be biscarbofuran-N,N'-disulfide ($CF-S_2-CF$), in agreement with spectroscopic data obtained from an authentic sample of this compound.

IV (114 mg) was subjected to further purification by TLC. The NMR spectrum was almost identical to that of III except for three singlets for the $N-CH_3$ protons (δ 3.38, 3.40 and 3.42). The mass spectrum of IV showed 4 molecular ion peaks at m/e 504 ($CF-S_2-CF$), 536 ($CF-S_3-CF$), 568 ($CF-S_4-CF$) and 600 ($CF-S_5-CF$). While it is possible that IV was still contaminated with a small amount of III, it is also possible that the molecular ion peak at 504 was the result of desulfurization by electron impact of a higher polysulfide. On the basis of NMR and mass spectral data, IV was concluded to have the following structure,



where n is 3 to 5.

The mixture containing V and VI (50 mg) was resubjected to TLC using hexanes-ether (1:1) as the developing solvent. V, mp 150-151°C proved to be identical to an authentic sample of carbofuran. VI stayed at the origin after development with 1:1 hexanes-ether and was not characterized owing to its small amount. Structures and TLC properties of the individual alteration products of DBSC formed in dichloromethane-acetic acid are given in Table 23.

Time Course for the Alteration of [Carbonyl-^{14}C]DBSC - In order to gain insight into the sequence of events taking place during the acid catalyzed alteration of DBSC, the breakdown of DBSC and formation of alteration products in 9:1 dichlormethane-acetic acid were monitored at short time intervals over a period of 120 hours.

Plots showing the relative amounts of each alteration product formed in dichloromethane-acetic acid at different time intervals are presented in Figure 2. The gradual disappearance of DBSC and concomitant formation of CF-S_n-NBu_2 (I), CF-S_2-CF (III), CF-S_n-CF (IV) and carbofuran (V) are clearly indicated. The major product after 48 hours was carbofuran (48%), followed by I (16%), III (10%) and IV (3.3%). During the first 84 hour period, the combined amount of III and IV (biscarbofuran polysulfides) was slightly less than the amount of I at each time interval but the relative amounts were reversed after 96 and 120 hours. During the early period of the kinetic study, significantly less IV was formed compared to III, e.g., 2.1% IV and 7.9% III after 24 hours. However, the rate of formation of IV increased more rapidly than the rate of formation of III and after 96 hours 7.1% IV was present compared to 10.9% for III. These results suggest that I, formed initially from DBSC, was being converted into IV.

The rate of DBSC decomposition into the different alteration products was first-order in DBSC with a first order rate constant for the disappearance of DBSC of 0.027 h^{-1}.

Time Course for the Alteration of [Dibutylamino-^{14}C]DBSC - An analogous study was conducted to examine the acid-catalyzed breakdown and formation of alteration products of [dibutylamino-^{14}C]DBSC in dichloromethane-acetic acid (9:1). The principal products were dibutylamine (VII, 51%) and I (16%), along with 23% of the starting DBSC. At least 6 minor unknown products (total 8.4%) also were detected.

Plots showing the relative amounts of products formed from [dibutylamino-^{14}C]DBSC in dichloromethane-acetic acid at different time intervals are shown in Figure 3. The plots reveal the gradual disappearance of DBSC and appearance of dibutylamine and CF-S_n-NBu_2 (I). As expected, the degradation of DBSC was first-order. It is noteworthy that evidence was not found for the conversion of the dibutylamino-labeled DBSC to Bu_2N-S_n-NBu_2. In contrast, CF-S_2-CF (III) and CF-S_n-CF (IV) were significant products from carbonyl-labeled DBSC under the same conditions.

Reversed-Phase TLC of Alteration Products - CF-S_n-NBu_2 ($n \geq 2$) and CF-S_n-CF ($n \geq 3$) which gave distinct single spots by silica gel TLC with a wide variety of developing solvents were separated into a number of components by reversed-phase TLC (see Figure 4). CF-S_n-NBu_2 was separated into at least 8 components (A-H) and CF-S_n-CF into at least 7 components (a-g). Compounds A-E proved to be CF-S_n-NBu_2 analogs with n=2-6 and compounds a-d were CF-S_n-CF analogs with n=3-6 (see next section). The R_f values of G and H corresponded to the bis(di-n-butylamino)-polysulfides which may have been formed during the workup procedure. Figure 1 reveals that CF-S-CF and CF-S_2-CF also were separable from CF-S_n-CF by reverse-phase TLC.

Identification of CF-S_n-NBu_2 Components - Preparative KC_{18} reversed-phase TLC of 70 mg CF-S_n-NBu_2 using acetonitrile as the developing solvent yielded four pure products, i.e., 36 mg A (R_f 0.49-0.56), 13 mg B (R_f 0.42-0.47), 7 mg C (R_f 0.35-0.40), and 3.5 mg D (R_f 0.30-0.34). Except for the N-CH_3

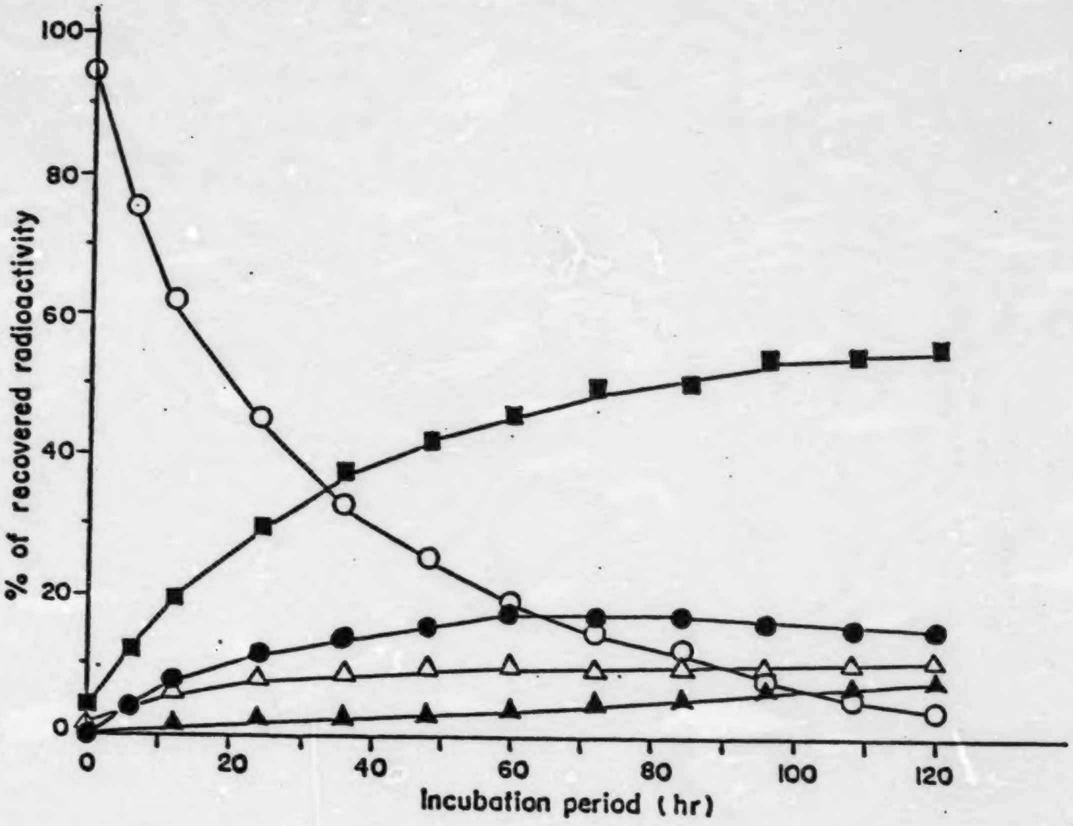


FIGURE 2

Plots showing the disappearance of [carbonyl-^{14}C]DBSC and formation of alteration products in dichloromethane-acetic acid (9:1). (O) DBSC, (■) carbofuran, (●) CF-S_n-NBu_2 (n=2-4), (△) CF-S_2-CF, (▲) CF-S_n-CF (n=3-5).

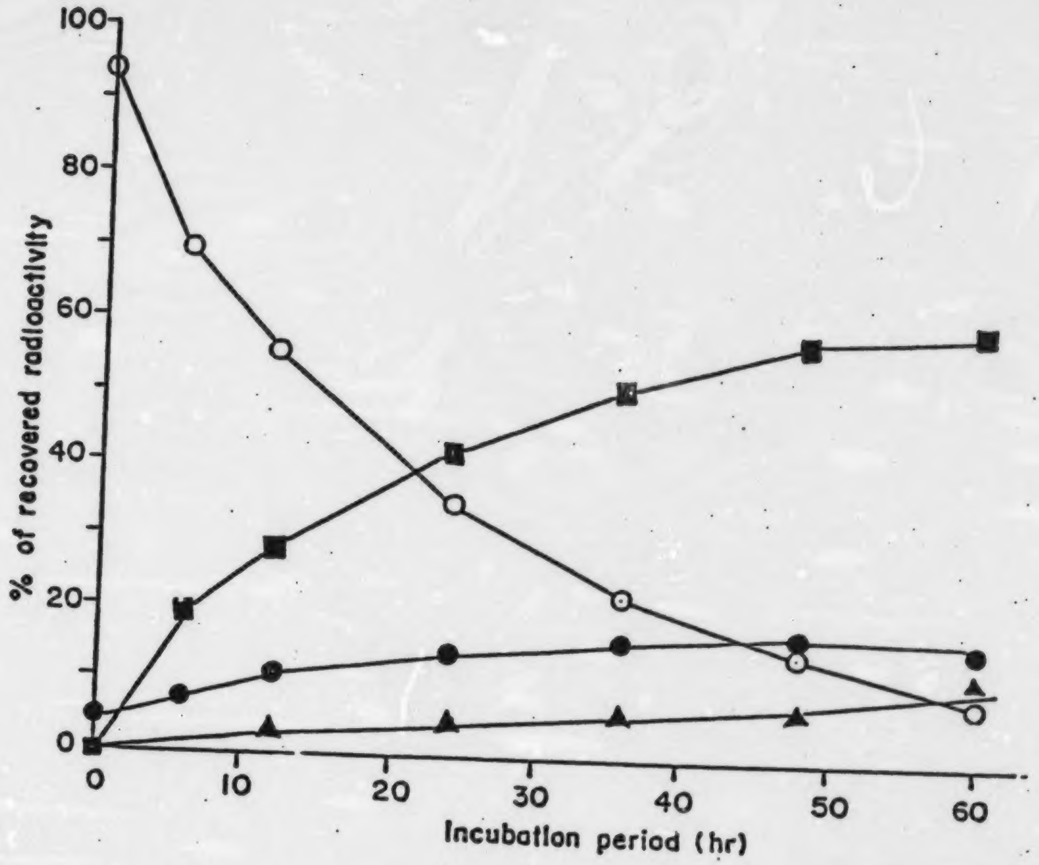


FIGURE 3

Plots showing the disappearance of [dibutylamino-^{14}C]DBSC and formation of alteration products in dichloromethane-acetic acid (9:1) (0) DBSC, (■) carbofuran, (●) CF-S_n-NBu_2 (n=2-4), (▲) unknowns.

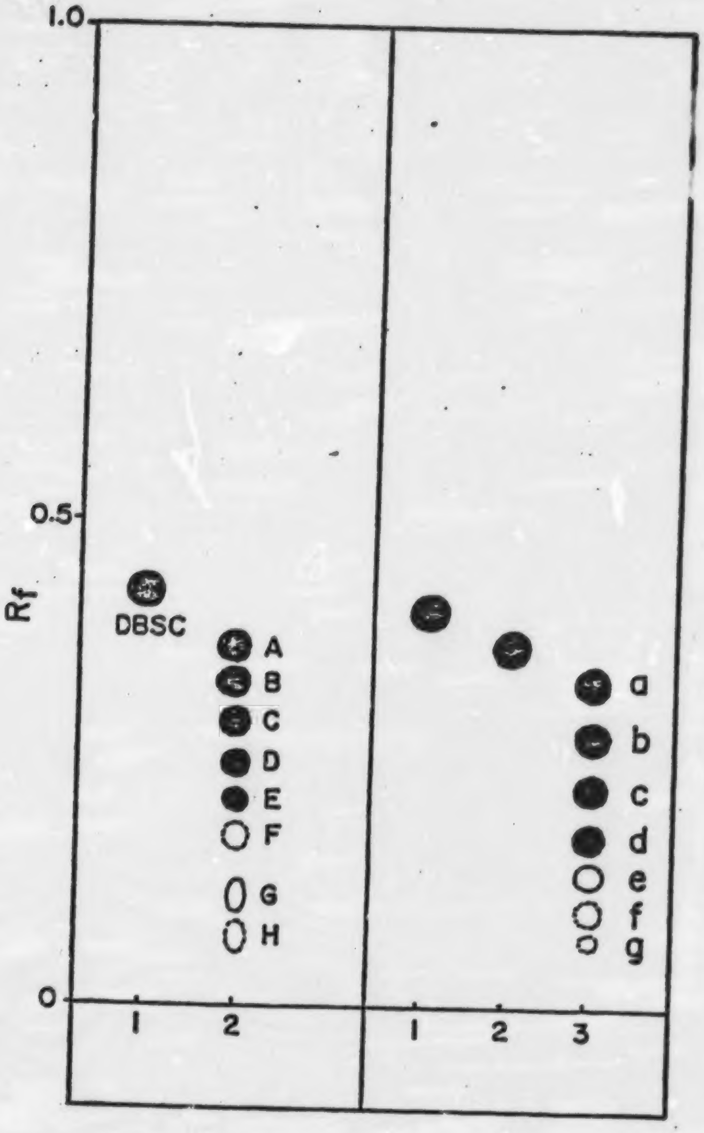


FIGURE 4

KC$_{18}$ reversed-phase thin-layer chromatograms of CF-S$_n$-NBu$_2$ (left) and CF-S$_n$-CF (right). (Left) 1: DBSC (CF-S-NBu$_2$); 2: CF-S$_n$-NBu$_2$ (n=2+3+...+n); solvent: acetonitrile. (Right) 1: CF-S-CF; 2: CF-S$_2$-CF; 3: CF-S$_n$-CF (n=3+4+...+n); solvent: acetonitrile-water (4:1) (two migrations) protons, the NMR spectra of A, B, C and D were identical to the spectrum of the polysulfide mixture CF-S$_n$-NBu$_2$. Earlier work showed a multiplet for the N-CH$_3$ protons of CF-S$_n$-NBu$_2$ at δ 3.3-3.42 (TMS, CDCl$_3$). In contrast, the N-CH$_3$ absorptions for A, B, C, and D were distinct singlets in the 3.3-3.4 p.p.m. region, indicating each to be a single compound. The mass spectrum of each compound indicated a molecular ion peak of 412 for A, 444 for B, 476 for C, and 508 for D. These molecular ion peaks were all observed in the mass spectrum of the polysulfide mixture CF-S$_n$-NBu$_2$. From NMR and mass spectral data the following structural assignments were made: A, CF-S$_2$-NBu$_2$; B, CF-S$_3$-NBu$_2$; C, CF-S$_4$-NBu$_2$; and D, CF-S$_5$-NBu$_2$.

Components E and F were isolated in trace amounts and appeared to be contaminated with material from the reversed-phase TLC plates. Definitive NMR or mass spectral data for these components could not be obtained. However, based on reversed-phase TLC properties of these compounds, E and F are tentatively considered to be CF-S$_6$-NBu$_2$ and CF-S$_7$-NBu$_2$, respectively.

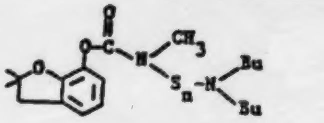
Data summarizing the TLC and spectroscopic properties of the polysulfide derivatives of DBSC are presented in Table 24.

Identification of CF-S$_n$-CF Components - Preparative reversed-phase TLC of 78 mg CF-S$_n$-CF gave at least 5 different products: 36 mg a (R$_f$ 0.46-0.53), 16 mg b (R$_f$ 0.37-0.43), 2.3 mg c (R$_f$ 0.31-0.36), 1 mg d (R$_f$ 0.24-0.28) and 2.0 mg e (Rf 0.09-0.22). Analytical reversed-phase TLC of each of these products showed that a, b, c and d were single component products. As in the case of the individual components of CF-S$_n$-NBu$_2$, the NMR spectra of a, b, c and d were identical to the spectrum of the polysulfide mixture CF-S$_n$-CF, except for the signals for the N-CH$_3$ protons. In contrast to the multiplet observed previously for the N-CH$_3$ protons (δ 3.37-3.44) for CF-S$_n$-CF distinct singlets in the same region were observed for a, b, c and d. Reversed-phase TLC and NMR spectra therefore indicated a, b, c and d to be single component products. The mass spectrum of each compound showed a molecular ion peak of 536 for a, 568 for b, 600 for c, and 632 for d. From TLC, NMR and mass spectral data, a was assigned the structure CF-S$_3$-CF, b CF-S$_4$-CF, c CF-S$_5$-CF and d CF-S$_6$-CF. Components e, f, and g were small amounts and were also contaminated with material from the TLC plates, and a definitive NMR spectrum could not be obtained. As in the case of E and F, e, f, and g are believed to be CF-S$_7$-CF, CF-S$_8$-CF, and CF-S$_9$-CF on the basis of their TLC behavior.

Data summarizing the TLC and spectroscopic properties of CF-S$_n$-CF and its components are given in Table 25.

Kinetic and Product Analysis of the Acid-Catalyzed Alteration of [Carbonyl-^{14}C]DBSC - [Carbonyl-^{14}C]DBSC was allowed to stand in 9:1 dichloromethane-acetic acid for 120 hours and the breakdown of DBSC and formation of alteration products were monitored at 24 hour intervals. The amounts of the different products formed from DBSC in the dichloromethane-acetic acid mixture at 24 hour intervals are given in Table 26. After 24 hours only 50% of the recovered radioactivity was present as the parent material which

TABLE 24. SUMMARY ON STRUCTURE AND CHEMICAL PROPERTIES OF EACH POLYSULFIDE DERIVATIVE OF DBSC.

Compound	R_f value TLC system[a]					MS	NMR	
	I	II	III	Structure	Abbreviation	Molecular ion peak (m/e)	N-CH$_3$ peak δ (ppm)	$\mathrm{N}\langle^{\mathrm{CH_2}}_{\mathrm{CH_2}}$ δ (ppm)
DBSC	0.43	0.43	0.61	n=1	-	380	3.35	3.17
CF-S_n-NBu_2	0.49	-	-	n=2+3+...+n	CF-S_n-NBu_2	-	3.33-3.42	2.95
A	0.49	0.37	0.54	n=2	CF-S_2-NBu_2	412	3.35	2.95
B	0.49	0.33	0.49	n=3	CF-S_3-NBu_2	444	3.38	2.95
C	0.49	0.30	0.43	n=4	CF-S_4-NBu_2	476	3.41	2.95
D	0.49	0.26	0.37	n=5	CF-S_5-NBu_2	508	3.42	2.95
E	0.49	0.21	0.32	n=6[b]	CF-S_6-NBu_2[b]			
F	0.49	0.11	0.17	n=7[b]	CF-S_7-NBu_2[b]			

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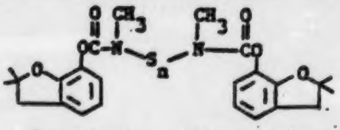
[a] TLC and solvent system: (I) silica gel TLC, hexanes-ether (7:3); (II) reversed-phase silica gel TLC, acetonitrile; (III) reversed-phase silica gel TLC, acetonitrile (2 migrations).

[b] Tentatively assigned.

TABLE 25. SUMMARY ON STRUCTURE AND CHEMICAL PROPERTIES OF POLYSULFIDE DERIVATIVES OF BISCARBOFURAN DISULFIDE

Compound	R_f value TLC system[a]				MS Molecular ion peak	NMR N-CH_3 peak
	I	II	Structure	Abbreviation	(m/e)	δ (ppm)
DBSC	0.43	0.23	-	-	380	3.35
CF-S_2-CF	0.26	0,39	n=2	CF-S_2-CF	504	3.42
CF-S_n-CF	0.23	-	n=3+4+...n			3.38~3.45
a	0.23	0.37	n=3	CF-S_3-CF	536	3.42
b	0.23	0.30	n=4	CF-S_4-CF	568	3.39
c	0.23	0.25	n=5	CF-S_5-CF	600	3.42
d	0.23	0.21	n=6	CF-S_6-CF	632	3.44
e	0.23	0.16	n=7[b]	CF-S_7-CF[b]		
f	0.23	0.14	n=8[b]	CF-S_8-CF[b]		
g	0.23	0.11	n=9[b]	CF-S_9-CF[b]		
Carbofuran	0.10	0.71	-	CF	221	2.82

[a] TLC and solvent system: (I) silica gel TLC, hexanes-ether (7:3); (II) reversed-phase silica gel TLC, acetonitrile-water (9:1).

[b] Tentatively assigned.

TABLE 26. ALTERATION OF [CARBONYL-^{14}C]DBSC IN DICHLOROMETHANE-ACETIC ACID (9:1) MIXTURE AFTER DIFFERENT INCUBATION PERIODS

Compound	Code	Percentage of total DBSC (hours)				
		24	48	72	96	120
DBSC		49.6	30.6	18.9	11.7	6.8
Carbofuran		27.4	39.9	47.1	52.4	56.5
$CF\text{-}S_n\text{-}NBu_2$ ($n \geq 2$)						
$CF\text{-}S_2\text{-}NBu_2$	A	7.8	11.7	13.4	13.6	13.0
$CF\text{-}S_3\text{-}NBu_2$	B	1.1	2.3	2.7	3.3	3.7
$CF\text{-}S_4\text{-}NBu_2$	C	0.2	0.3	0.5	0.6	0.7
$CF\text{-}S_5\text{-}NBu_2$	D	0.1	0.1	0.2	0.2	0.2
$CF\text{-}S_6\text{-}NBu_2$	E	<0.1	<0.1	0.1	0.1	0.1
Subtotal:		9.2	14.4	16.9	17.8	17.7
$CF\text{-}S_n\text{-}CF$ ($n \geq 2$)						
$CF\text{-}S_2\text{-}CF$		6.1	7.0	8.3	8.2	8.6
$CF\text{-}S_3\text{-}CF$	a	2.2	2.9	3.0	4.3	4.0
$CF\text{-}S_4\text{-}CF$	b	0.2	0.5	1.1	1.6	2.0
$CF\text{-}S_5\text{-}CF$	c	0.4	0.4	0.7	0.7	0.7
Subtotal:		8.9	10.8	13.1	14.8	15.3
Unknowns		5.0	4.4	4.2	3.4	3.6
Total:		100.1	100.1	100.2	100.1	99.9

gradually decreased during the 120 hour test period. The amounts of carbofuran and CF-S$_n$-CF (n=2-5) gradually increased while CF-S$_n$-NBu$_2$ (n=2-6) reached a maximum at 96 hours after which it decreased. These observations are in agreement with results reported in a preceding section in which silica gel TLC was used as a means of separating products. Of the polysulfide derivatives of DBSC, i.e., CF-S$_n$NBu$_2$, CF-S$_2$-NBu$_2$ was the major product (7.8-13%) throughout the entire test period, followed by CF-S$_3$-NBu$_2$ (1.1-3.7%) and CF-S$_4$-NBu$_2$ (0.2-0.7%). CF-S$_2$-NBu$_2$ reached a maximum after 72 hours and remained at a constant level for another 48 hours while CF-S$_3$-NBu$_2$ and CF-S$_4$-NBu$_2$ gradually increased throughout the test period.

Of the biscarbofuran polysulfide derivatives, CF-S$_2$-CF was the major product (6.1-8.6%) at each time interval, followed by CF-S$_3$-CF (2.2-4.0%), CF-S$_4$-CF (0.2-0.3%) and CF-S$_5$-CF (0.4-0.7%). Most of these derivatives showed a gradual increase over the test period. Of interest is the constant 3:2 ratio between the amount of carbofuran and total amounts of polysulfide derivatives (CF-S$_n$-NBu$_2$ + CF-S$_n$-CF) which was observed during the 120 hour study.

Toxicological Properties of the Alteration Products - Data for the toxicity of several of the acid-catalyzed alteration products of DBSC to the house fly and white mouse are given in Table 27. With the exception of CF-S$_2$-CF, all of the compounds showed good insecticidal activity against the house fly, comparable in activity to carbofuran. For reasons which are not clear, CF-S$_2$-CF was nontoxic to house flies at 250 µg/g. In contrast to house fly toxicity, all of the alteration products were significantly less toxic to the white mouse than the parent methylcarbamate, carbofuran.

Summary - In dichloromethane-acetic acid DBSC experienced N-S bond cleavage resulting in variety of reaction products in addition to carbofuran. These were the polysulfide derivatives of DBSC (I), biscarbofuran-N,N'-disulfide (III) and polysulfides (IV), and dibutylamine.

Analysis of the individual products obtained from carbonyl- and dibutylamino-^{14}C labeled DBSC showed that the amount of CF-S$_n$-NBu$_2$ (I) gradually increased to a steady state level after 60-72 hours while the amounts of CF-S$_2$-CF (III) and CF-S$_n$-CF (IV) continued to increase throughout the entire reaction period of 120 hours. This would suggest that CF-S$_n$-NBu$_2$ is an intermediate and is slowly transformed into CF-S$_2$-CF and CF-S$_n$-CF.

Silica gel KC$_{18}$ reversed-phase TLC provided a simple and convenient method for separating the individual components in the polysulfide mixtures of DBSC and biscarbofuran disulfide which previously could not be separated by conventional silica gel TLC. CF-S$_n$-NBu$_2$, the polysulfide of DBSC, was separated into at least 8 components and CF-S$_n$-CF, the biscarbofuran derivatives, was separated into 7 components. In the reversed-phase separation of these components, general correspondence between R$_f$ values and the number of sulfur atoms was observed, i.e., R$_f$ values decreased as the number of sulfur atoms increased.

TABLE 27. TOXICITY OF THE ALTERATION PRODUCTS AND RELATED COMPOUNDS AGAINST HOUSE FLIES AND MICE

Compound	Toxicity	
	House fly LD_{50} (μg/g)	Mouse LD_{50} (mg/kg)
$CF-S_n-NBu_2$ ($n \geq 2$)	7.5-15.0	127^{a} $(74-155)^{c}$
$CF-S_2-NBu_2$	9.8	-
DBSC ($CF-S-NBu_2$)	7.8	129^{a} $(107-148)^{c}$ 74^{b} $(59-124)^{c}$
$CF-S_n-CF$ ($n \geq 3$)	15.0	$>100^{b}$
$CF-S_3-CF$	12.2	-
$CF-S_2-CF$	>250	$>100^{b}$
CF-S-CF	24.0	$50-100^{d}$
Carbofuran	11.0	19.4^{a} $(16.2-24.3)^{c}$ 11.1^{b} $(9.8-12.4)^{c}$

[a] Compound was dissolved or suspended in corn oil. [b] Compound was dissolved or suspended in propylene glycol. [c] 95% confidence limit. [d] Value from Fahmy et al. (1974).

Although the overall structure of each component in CF-S_n-NBu_2 and CF-S_n-CF was established by NMR and mass spectral analysis, it was not possible to determine the exact nature of the polysulfide moiety. Mass spectra of these components provided information relating to the number of sulfur atoms but did not indicate whether the sulfur atoms existed in a straight chain or were branched, or were part of a ring system. Polysulfides of elemental sulfur have been shown to exist in all three forms[5].

The poor insecticidal activity of CF-S_2-CF was unexpected in light of the good activity observed for the other derivatives. A good explanation for the poor activity of this compound is presently not available. It is possible that the disulfide linkage in this compound is uniquely different from the other polysulfide derivatives so as to prevent or reduce formation of carbofuran in the house fly.

3. References.

1. Pellegrini,G., R. Santi, J. Agric. Food Chem. 20 944 (1972).

2. Takade, D. Y., M. S. Seo, T. S. Kao, T. R. Fukuto, Arch. Environ. Contam. Toxicol. 5, 63 (1976).

3. Metcalf, R. L., T. R. Fukuto, C. Collins, K. Borck, S. Abu-El-Aziz, J. Agric. Food Chem. 16, 300 (1968).

4. Getzin, L. W., Environ. Entomol. 2, 461 (1973).

5. Meyer, B., Chem. Rev. 76, 367 (1976).

E. EFFECT OF IMPURITIES ON THE POTENTIATION OF ORGANOPHOSPHORUS INSECTICIDES

1. Identification and Toxicological Properties of Impurities.

Introduction - Numerous examples are known where enhancement of toxicity has been observed when a combination of chemicals is administered to a mammal. Perhaps the first example of this is the potentiation of the mammalian toxicity of malathion by EPN which was observed over 20 years ago and subsequently reported by Frawley et al.[1] Although the above example cites a case of potentiation between two insecticides, potentiation also is caused by minor impurities present in technical organophosphorus insecticides. Pellegrini and Santi[2] described the potentiating activity of a variety of simple trimethyl phosphorothioate and phosphorodithioate esters on the mammalian toxicity of such safe organophosphorus insecticides as phenthoate and malathion. This section is concerned with the identification of the major impurities which are commonly present or may be developed upon storage in technical malathion and acephate, and the evaluation of the effect of the impurities on the mammalian toxicity of the purified insecticides.

Toxicity of Purified and Technical Malathion - As shown in Table 1, technical malathon (∿95% pure) varied in acute rat oral LD_{50} from about 1,400 to 1,600 mg/kg, while the purified materials gave values of about 9,500 (99.3% pure) and 12,500 (recrystallized) mg/kg. In the case of mice, technical malathion varied in acute LD_{50} from 1,750 to 2,050 mg/kg, while purified materials gave values of 3,000 (99.3% pure) and 3,600 (recrystallized) mg/kg. No toxicity difference was observed between the purified and technical malathion following topical application to house flies. These results indicated that impurities present in technical malathion potentiated the toxicity of malathion to rats and mice.

Isolation and Identification of Impurities in Technical Malathion - A sample of technical malathion (95% pure) was initially examined qualitatively by TLC and GC. At least 4 impurities less polar than malathion (referred to as nonpolar impurities or *NPI*) were detected by TLC using benzene as the developing solvent, and at least 8 impurities more polar than malathion (referred to as polar impurities or *PI*) were detected using benzene-ethyl acetate (9:1 and 1:1) as solvent. At least 11 impurities were observed by GC.

The individual impurities which were separated by column chromatography or by TLC were identified by cochromatography with authentic samples, and by NMR and mass spectroscopy.

The structures of the various impurities identified in technical malathion along with amounts detected, TLC and GC characteristics, are presented in Table 2. The presence of these compounds was reaffirmed by GC-MS analysis of technical malathion. GC-MS also revealed the presence of trace amounts of *O*,*O*,*O*-trimethyl phosphorothioate and *O*,*O*-dimethyl phosphonothionate in technical malathion.

TABLE 1. TOXICITY OF PURIFIED AND TECHNICAL MALATHION TO RATS, MICE AND HOUSE FLIES

Malathion	LD_{50} Rat (mg/kg)	LD_{50} Mouse (mg/kg)	LD_{50} House Fly (μg/g)
Purified I[a]	-	3,200	29.0
Purified II[b]	9,500	3,000	-
Purified III[c]	12,500	3,600	-
Technical I[d]	1,500	1,850	29.0
Technical II[e]	-	1,850	-

[a] Purified by column chromatography.

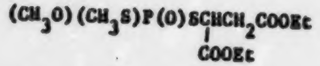
[b] Obtained from the American Cyanamid Company (99.3% pure).

[c] Purified by recrystallization.

[d] Obtained from the EPA Battelle Repository.

[e] Obtained from the American Cyanamid Company (95% pure).

TABLE 2. STRUCTURES, TLC AND GC PROPERTIES OF IMPURITIES PRESENT IN TECHNICAL MALATHION.

Impurity	Structure	Amount Present (%)	Color DBQ	TLC R_f a/				Rel. retention times (sec)		
				I	II	III	IV	5% OV-101 b/	4% AN-600 c/	4% AN-600 d/
A	$(CH_3O)_2P(S)SCH_3$	1.1	red	0.68	0.86	-	0.55	15	-	33
B	$(CH_3O)_2P(S)SP(S)(OCH_3)_2$	~0.5	red	0.67	0.86	-	0.46	84	-	-
E	$HSCH(COOEt)CH_2COOEt$	trace	brown	0.47	0.82	-	0.0	-	-	-
F	$(CH_3O)_2P(S)SCH(COOEt)CH_2COOCH_3$ and $(CH_3O)_2P(S)SCH(COOCH_3)CH_2COOEt$	>0.1	red	0.45	0.81	-	-	147	-	-
G	$[SCH(COOEt)CH_2COOEt]_2$	>0.3	orange	0.43	0.80	-	-	-	-	-
H-1	$S[CH(COOEt)CH_2COOEt]_2$	>0.5	light orange	0.37	0.79	-	-	-	-	-
J	 $(CH_3O)(CH_3S)P(O)S\underset{COOEt}{\underset{\vert}{CH}}CH_2COOEt$	0.2	yellow to brown	0.12	0.56	-	-	216	-	-
L	$(CH_3S)_2P(O)OCH_3$	0.003	yellow	0.07	0.40	-	-	-	54	100
M	$(CH_3O)_2P(S)SCH(COOEt)CH_2COOH$	0.6-0.8	red	0.05	0.17	0.50	-	-	-	-
N	$(CH_3O)_2P(S)SCH(COOH)CH_2COOEt$	0.1-0.15	red	0.05	0.05	0.45	-	-	-	-
O	$(CH_3O)_2P(O)SCH_3$	0.04	yellow	0.07	0.31	-	-	-	30	48

a/ Solvent systems: I, benzene-ethyl acetate (9:1); II, benzene-ethyl acetate (1:1); III, benzene-ethyl acetate-acetic acid (50:50:3); IV, hexane-benzene (1:1).

b/ Column temperature, 190°; port temperature, 220°.

c/ Column temperature, 170°; port temperature, 200°.

d/ Column temperature, 150°; port temperature, 185°.

Toxicological Evaluation of Malathion Impurities - Of the impurities identified in technical malathion, eight were examined for their toxicological effects when added to purified malathion. Impurity B or bisdimethoxyphosphinothioyl sulfide was not examined since this material, synthesized by an independent method, itself contained a number of impurities. Table 3 contains toxicological data for the effect of the various impurities on the toxicity of purified malathion to the rat and mouse. Included also are data for the toxicity of the impurity itself to each animal (indicated as 100% impurity in the table). It is evident from the data that O,O,S-trimethyl phosphorodithioate (A), S-methyl isomeride of malathion (J), O,S,S-trimethyl phosphorodithioate (L), O,O,S-trimethyl phosphorothioate (O) and malathion mono-acid all increase the toxicity of purified malathion to mice and rats. Based on the log dose-probit response (LD-p) lines for toxicity determination and the amount of each impurity added to malathion, it can be concluded that the increase in toxicity is attributable to potentiating and not to additive effects. The potentiation of phenthoate and malathion by A, L, and O was previously reported by Pellegrini and Santi[2].

Potentiating effects were decidedly greater in the rat compared to the mouse. This becomes apparent when the toxicity ratios (relative to the toxicity of purified malathion) are compared. Of the various impurities examined, L showed the highest potentiating activity to rats, followed by the S-methyl isomeride of malathion (J), and A. In light of the relatively large amounts of A and J in technical malathion and their potentiating activity, these two impurities undoubtedly contribute to the higher toxicity of technical malathion. Although L showed the highest potentiating activity, it was present to only a minor extent in technical malathion (0.003%).

Toxicity of Purified and Technical Acephate - As shown in Table 4, technical acephate varied in acute oral LD_{50} from 1,580 to 2,100 mg/kg for rats and 480 to 520 mg/kg for mice, while purified (recrystallized) acephate gave values of 1,350 to 1,650 for rats and 395 to 450 mg/kg for mice. Further, addition of the concentrated impurities (5% by weight), obtained from the mother liquor after recrystallization of technical acephate, to purified acephate decreased the toxicity of the pure material by about 22%. No toxicity difference was observed between the purified and technical acephate following topical application to house flies. These results suggest that impurities present in technical acephate antagonize mammalian toxicity of acephate.

Isolation and Identification of Impurities in Technical Acephate - Analytical silica gel TLC showed that technical acephate contains a number of impurities but in very small amounts. Therefore, the technical material was recrystallized and the mother liquor was used for isolation of the impurities. The individual impurities were separated by column chromatography and TLC and their structures were determined by cochromatography, NMR and mass spectroscopy. The structures of the various impurities identified in technical acephate along with amounts detected, TLC and GC characteristics are presented in Table 5.

TABLE 3. THE EFFECT OF MALATHION IMPURITIES ON THE TOXICITY OF PURIFIED MALATHION TO MICE AND RATS

	Mouse toxicity			Rat toxicity		
Impurity	% added impurity	LD_{50} (mg/kg)	Ratio[a]	% added impurity	LD_{50} (mg/kg)	Ratio[a]
A	5.0	2,530	1.27	0.5	6,900	1.8
	100[b]	1,850	-	1.0	3,200	3.9
				5.0	2,450	5.0
				100	660	-
E	5.0	4,230	0.76	2.0	>8,000	-
G	5.0	4,100	0.78	2.0	>8,000	-
J	0.5	2,350	1.37	0.05	4,400	2.9
	2.0	1,150	2.78	0.1	3,100	4.0
	100	330	-	0.5	1,975	6.3
				1.0	1,920	6.7
				2.0	1,250	10.0
				100	120	-
L	1.0	2,230	1.42	0.05	3,100	4.2
	2.0	2,060	1.57	0.2	2,100	5.9
	100	400	-	0.5	1,775	7.2
				100	110	-
M, N	5.0[c]	2,720	1.18	2.0[d]	8,900	1.4
O	1.0	3,080	1.04	0.2	4,100	3.0
	100	530	-	1.0	2,900	4.4
				100	260	-

[a] LD_{50} of recrystallized malathion (rat 12,500 mg/kg, mouse 3,200 mg/kg) divided by the LD_{50} of recrystallized malathion plus impurity.

[b] The value 100% denotes that impurity was used alone.

[c] Malathion β-mono-acid was used.

[d] An approximately 1:1 mixture of M and N was used.

TABLE 4. TOXICITY OF PURIFIED AND TECHNICAL ACEPHATE TO RATS, MICE AND HOUSE FLIES

Acephate	LD_{50} Rat (mg/kg)	LD_{50} Mouse (mg/kg)	LD_{50} House Fly (µg/g)
Purified (recrystallized)	1,480	425	1.65
Technical	1,800	500	1.68
Purified + 5% conc. imp.[a]	-	520	1.30

[a] The concentrated impurities remaining in the mother liquor after recrystallization of acephate were added to purified acephate.

TABLE 5. STRUCTURES, TLC AND GC PROPERTIES OF IMPURITIES PRESENT IN TECHNICAL ACEPHATE.

Impurity	Structure[a]	Amount Present (%)	Color DBQ	TLC R_f[b] A	TLC R_f[b] B	TLC R_f[b] C	TLC R_f[b] D	Rel. retention times (sec) 4% AN-600[c]	Rel. retention times (sec) 4% AN-600[d]
IV	$(CH_3O)_2P(S)NH_2$	0.07	red	0.65	0.90	1.0	-	42	27
V	$(CH_3O)(CH_3S)P(S)NH_2$	trace	red	0.55	0.88	-	-	-	60
VI	$(CH_3O)_2P(S)NHCOCH_3$	0.44	red	0.48	0.84	1.0	-	150	78
VII	$(CH_3O)_2P(O)SCH_3$	0.20	yellow	0.31	0.79	0.80	0.62	39	27
VIII	$^{+}M^{-}SP(O)(OCH_3)NHCOCH_3$	trace	red	0	0.53	0.59	-	-	-
X	$^{+}M^{-}OP(O)(SCH_3)OCH_3$	2.0	yellow	0	0.22	0.54	0.12	-	-
XI	$^{+}M^{-}OP(O)(SCH_3)NHCOCH_3$	trace	yellow	0	0.18	0.47	0.05	-	-

[a] M^+ is an unknown cation.

[b] Solvent systems: A, benzene-ethyl acetate (1:1); B, acetonitrile-methanol-H_2O (16:3:1); C, ethyl acetate-methanol (1:1); D, diethyl ether-isopropanol (2:3).

[c] Column temperature, 160°; port temperature, 205°.

[d] Column temperature, 185°; port temperature, 215°.

Toxicological Evaluation of Acephate Impurities - Table 6 contains toxicological data for the effect of impurities IV, VI, VII and X on the toxicity of purified acephate to mice. The toxicity of each impurity alone to mice also is included. As indicated in the table, IV and X had virtually no effect on the mouse toxicity of acephate. O,O,S-Trimethyl phosphorothioate (VII) caused a slight degree of potentiation, e.g., 1% VII added to purified acephate changed the LD_{50} from 570 to 460 mg/kg. In contrast, O,O-dimethyl N-acetylphosphoramidothioate (VI) added to purified acephate caused a significant decrease in mouse toxicity. As indicated in Table 5, VI was one of the major impurities present in technical acephate and it probably contributed to the lower mouse toxicity of the technical material compared to purified acephate.

Effect of Storage on the Toxicity of Technical Malathion and Acephate - The effect of storage of technical malathion and acephate on toxicity was examined at 20° and 40° for a period of 6 months. Table 7 provides data which show the change in toxicity of these two materials to mice and house flies during the 6 months storage period. Little change in malathion mouse toxicity was observed after storage at 20°, but a notable increase in toxicity was apparent after storage at 40°. GC analysis of technical malathion stored at 40° for 6 months showed a slight decrease in malathion content, from 95% to 94%, but this small difference was within experimental error. These results showed, however, that a minute change in malathion content and, therefore, the composition of technical malathion had a strong effect on its toxicity to mice. No change in toxicity to house flies was observed.

In contrast to technical malathion, mouse toxicity of technical acephate decreased with storage. For example, after 6 months storage, technical acephate was about two-thirds as toxic to mice as the original material. Toxicity to house flies, however, was virtually unchanged, indicating that the decrease in mouse toxicity was not attributable to acephate decomposition.

Impurities A and J were monitored in malathion and impurities IV, VI, VII, and methamidophos (O,S-dimethyl phosphoramidothioate) were monitored in acephate. The results showed that the amount of A decreased slightly during the storage period at both temperatures, while J increased about 2-fold after 6 months storage at 40°. In light of the strong potentiating activity of J, the higher concentration of J is in agreement with the increased toxicity of malathion to mice.

Photolysis of Malathion - Sunlight irradiation of recrystallized malathion, shown by GC to contain traces of O,O,S-trimethyl phosphorodithioate, malaoxon and malathion mixed esters, on glass or silicic acid surfaces for periods up to eight days led only to small changes in the relative intensities of the various components. However, rapid changes in composition did occur on irradiation with a medium pressure mercury lamp. After 1 hr on the glass surface, GC analysis indicated the formation of three new phosphorus containing compounds which eluted at 120°, 141° and 157°. On the basis of comparison of retention times with those of authentic materials, the compounds

TABLE 6. THE EFFECT OF ACEPHATE IMPURITIES ON THE TOXICITY OF PURIFIED ACEPHATE TO MICE

Impurity	% added impurity	Mouse LD_{50} (mg/kg)	Ratio[a]
IV	5.0	570	1.0
	100[b]	430	-
VI	1.0	720	0.79
	5.0	800	0.71
	100	1,150	-
VII	0.5	540	1.05
	1.0	460	1.25
	100	530	-
X	5.0	570	1.0
	100	>3,600	-

[a] LD_{50} of recrystallized (570 mg/kg) divided by the LD_{50} of recrystallized acephate plus impurity.

[b] The value 100% denotes that impurity was used alone.

TABLE 7. EFFECT OF STORAGE ON THE TOXICITY OF TECHNICAL MALATHION AND ACEPHATE TO MICE AND HOUSE FLIES.

Storage Period (months)	Temp °C	Malathion LD_{50}		Acephate LD_{50}	
		Mouse (mg/kg)	House Fly (µg/g)	Mouse (mg/kg)	House Fly (µg/g)
0	-	2,400	33.0	500	1.25
3	20	2,300	-	640	-
6	20	2,350	-	720	-
3	40	2,000	32.0	790	1.25
6	40	1,780	32.0	820	1.10

eluting at 120° and 141° were assigned as O,O,O,O-tetramethyl pyrophosphorodithioate and bis(dimethoxyphosphinothioyl) sulfide, respectively. Following irradiation for a further 3 hr, all three of the new components increased at the expense of malathion, which was judged by relative intensities to have undergone an approximate 40% reduction. Malaoxon also showed a slight increase, and traces of two new compounds eluting at 163° and 164° appeared. (Under these GC conditions malathion mixed esters, malathion, and malaoxon elute at 168°, 170° and approximately 178°, respectively). A similar result occurred on the silicic acid surface; however, the compound eluting at 157° was formed in only minor amounts and malaoxon formation was enhanced.

Fenthion Impurities - GC analysis of technical fenthion (96%) indicated 23 impurities, 9 of shorter and 14 of longer retention times than fenthion. Following GC-MS of impurity rich fractions obtained from either column chromatography or preparative GC, structures were assigned to eight compounds on the basis of their fragmentation patterns, with two, O,O,O-trimethyl phosphorodithioate and O,O,O,O-tetramethyl pyrophosphorodithioate, being confirmed by comparisons with authentic samples. Of the two other phosphorus containing impurities, one showed a molecular ion of 232 (50%) with significant fragment ions at m/e 125 (49%), 109 (100%), 93 (49%), 79 (50%), 63 (35%) and 47 (28%) and was assigned the structure O,O-dimethyl O-3-methylphenyl phosphorothioate. The fourth phosphorus containing compound and the only compound of longer retention time than fenthion which was identified by GC-MS showed a molecular ion at m/e 278 (8%), thereby suggesting it to be an isomer of fenthion. This impurity was assigned as O,S-dimethyl O-3-methyl-4-methylthiophenyl phosphorothioate.

Four other phosphorus containing impurities with retention times longer than fenthion have also been tentatively identified as the sulfoxide and sulfone of fenthion and the sulfoxide and sulfone of fenoxon on the basis of GC comparisons with authentic samples.

Toxicological Evaluation of Technical Fenthion - Technical fenthion (96%) had a rat acute oral LD_{50} value of 1025 mg/kg compared with 1100 mg/kg for material purified by column chromatography. This minor difference most likely reflects the small amount of fenoxon present in the technical material. The poisoning symptoms shown by the rats were those typical of cholinesterase inhibition.

Summary - A number of impurities present in technical malathion and acephate were isolated by column and thin-layer chromatography and the structures of 11 impurities in malathion and 7 impurities in acephate were determined by nuclear magnetic resonance, infrared and mass spectroscopy, and by gas and thin-layer chromatography. Several of the impurities potentiated rat toxicity of purified malathion and highest potentiating activity was observed with O,S,S-trimethyl phosphorodithioate and the S-methyl isomeride of malathion. Potentiating effects were significantly smaller in the mouse compared to the rat. Of the impurities isolated from acephate, O,O,S-trimethyl phosphorothioate showed slight potentiation of mouse toxicity when added to purified acephate, but an antagonizing effect was observed with O,O-dimethyl N-acetylphosphoramidothioate. Storage of technical malathion for 3 to 6 months at 40° resulted in materials which were noticeably more toxic to mice. In contrast, a significant reduction in mouse toxicity was observed after storage of technical acephate under the same conditions.

The mammalian safety of malathion has been attributed to the susceptibility of the carboethoxy groups to hydrolytic detoxication to the corresponding carboxylic acid derivative. This reaction, mediated by the carboxylesterases, evidently takes place in mammals at a rate faster than the activation reaction leading to malaoxon, the metabolite responsible for inhibition of the cholinesterase enzymes[3]. On the other hand, the low mammalian toxicity of acephate and related N-acylphosphoramidothioates has been explained on the basis of their ability to avoid hydrolytic activation to the corresponding phosphoramidothioate, i.e., to the more effective anticholinesterase methamidophos. If the assumption is made that impurities in malathion and acephate are able to interfere with esterase cleavage of the O-acyl or N-acyl linkages, respectively, then the impurities would be expected to have a potentiating effect on malathion but an antagonizing effect on acephate. The toxicological data were, in general, consistent with this prediction, indicating that esterases are involved in the potentiation or antagonism of malathion and acephate.

2. Mode of Potentiating Action of Impurities Present in Malathion.

Introduction - The preceding section described the potentiating action of several impurities which are present in technical malathion on the toxicity of malathion. Work also was undertaken to investigate the biochemical mechanism underlying the potentiating actions of these compounds. The results presented in this section provide in vitro and in vivo evidence that these compounds diminish the activities of the enzymes involved in malathion detoxification and also diminish the activity of serum cholinesterase.

The four impurities which were examined were O,O,S-trimethyl phosphorodithioate (1), O,O,S-trimethyl phosphorothioate (2), O,S,S-trimethyl phosphorodithioate (3) and the S-methyl isomeride of malathion (4).

In Vitro Inhibition of Carboxylesterase and Cholinesterase - The four impurities isolated from technical malathion were incubated in vitro with rat serum or with an enriched liver malathion carboxylesterase. The serum carboxylesterase and cholinesterase activities were diminished by compounds 2-4, but not by compound 1 (Table 8). When the liver carboxylesterase was used as the target enzyme, all four compounds diminished its activity (Table 8). From the concentrations of compounds 1-4 estimated to produce 50% inactivation of the target enzymes, the order of potency of the compounds would appear to be, $4 \gtrsim 3 > 2 > 1$.

Groups of rats were treated with various doses of compounds 1-4 to assess in vivo their potencies as inactivators of serum malathion carboxylesterase. The doses estimated to cause a 50% diminution of serum malathion carboxylesterase activity are given in Table 9. In agreement with the in vitro experiments, compounds 3 and 4 were the most potent of the inactivators.

TABLE 8. INACTIVATION OF ESTERASES BY MALATHION IMPURITIES IN VITRO IN THE RAT[a].

Compound	IC_{50} (95% confidence limits) (mM) Serum malathion carboxylesterase	Serum cholinesterase	Liver malathion carboxylesterase
1	>5	>5	0.5 (0.33-0.89)
2	0.9 (0.45-1.8)	1.6 (0.14-7.9)	0.11 (0.07-0.24)
3	0.04 (0.02-0.1)	0.17 (0.07-0.33)	0.002 (0.0007-0.005)
4	0.00045 (0.0002-0.001)	0.003 (0.0015-0.0053)	0.0001 (0.00001-0.001)

---

[a] Graded concentrations of compounds 1-4 were incubated with the target enzymes. After 1 hr incubation periods, the residual enzyme activities were assayed. The IC_{50} values and their 95% confidence limits were determined by linear regression analysis of the data (x = log concentration in millimolar; y = percentage inactivation in probits).

TABLE 9. IN VIVO INACTIVATION OF SERUM MALATHION CARBOXYLESTERASE BY MALATHION IMPURITIES IN THE RAT[a].

Compound	ID_{16} (mg/kg)	ID_{50} (mg/kg)	ID_{84} (mg/kg)
1	25	55	104
2	9	38.5	123
3	0.9	1.9	5.9
4	0.1	1.3	16

---

[a] Graded doses of compounds 1-4 were administered to groups of rats. Five groups of four rats were used to evaluate each compound. Blood samples were drawn from rats 4 hours after dosing with compound 1 (the latter compound proved to be slower acting). The malathion carboxylesterase activities remaining in the sera were assayed and the values were estimated from log-probability plots.

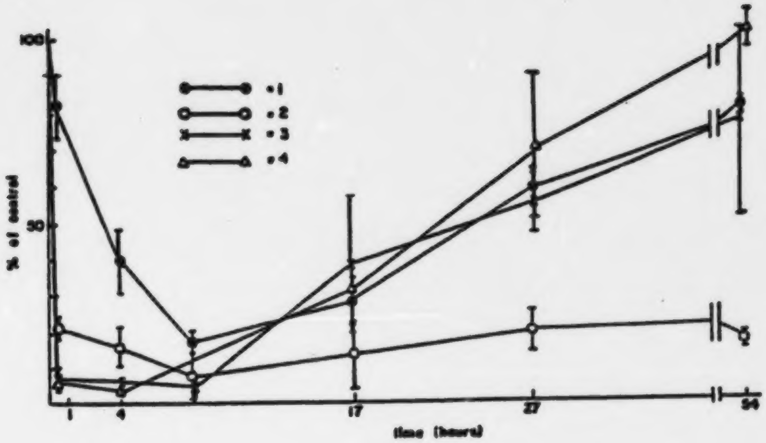


FIGURE 1

Variation of serum malathion carboxylesterase activity with time after dosing with compounds 1-4. Four groups of six rats received the test compounds at time zero, and blood samples were obtained from three of six rats/group at various times after dosing. Residual malathion carboxylesterase activities in the sera were assayed. Results for each time point were averaged and expressed as percentages of activity measured in a control group. The mean ± SD obtained at each point is shown in the figure.

Following a single dose of a selected test compound, the variation of rat serum malathion carboxylesterase was monitored over an interval of 54 hours (Fig. 1). The onset of action of compounds 2-4 was relatively rapid, with maximal or near maximal diminutions observed in the 30-min serum samples. In contrast, compound 1, not effective as an *in vitro* inactivator, required several hours to attain its maximal effect *in vivo*, suggesting that this compound requires metabolic activation. At the end of the 54-hr monitoring period, serum malathion carboxylesterase activity had recovered to greater than 75% of the normal value in animals treated with compounds 1, 3, and 4. However, the diminution produced by compound 2 was relatively long-lived—recovery of activity during the monitoring period was not statistically significant (Table 10).

TABLE 10. RECOVERY OF SERUM MALATHION CARBOXYLESTERASE ACTIVITY IN THE RAT[a].

Compound	Dose (mg/kg)	Rate of recovery (%/hr)	Regression coefficient (R)	Significance (∫)
1	104	1.39	0.96	<0.05
2	104	0.066	0.34	NS[b]
3	25	1.44	0.95	<0.05
4	105	1.95	0.95	<0.05

---

[a] The rate of recovery of serum malathion carboxylesterase activity in animals that received a single dose of inactivator was estimated from the data presented in Fig. 2. The slopes of the recovery lines were estimated by linear regression using as coordinate points all data points obtained after the times of maximal enzyme inhibition. The regression coefficient *R* was obtained and its significance, i.e., the dependence of enzyme activity on time, was assessed using the *t* test.

[b] Not significant.

Single dose studies demonstrated that compounds 1-4 could diminish rat liver malathion carboxylesterase activity and also rat serum cholinesterase activity *in vivo* (Table 11, Fig. 2). The serum cholinestrase activity was monitored with time, as in the malathion carboxylesterase study. From the trend of the recovery lines (Fig. 2), diminution of serum cholinesterase activity appeared transient when compounds 1, 3, or 4 were the effectors but relatively long-lived when compound 2 was the effector.

TABLE 11. IN VIVO INHIBITION OF LIVER MALATHION CARBOXYLESTERASE BY MALATHION IMPURITIES IN THE RAT[a].

Compound	Dose (mg/kg)	Percentage control activity
1	104	28.4
2	104	27.0
3	25	12.3
4	105	4.4

---

[a] Groups of four rats received the test compounds at the doses indicated. Rats were sacrificed 17 hours after dosing and the malathion carboxylesterase activities remaining in the liver homogenates were assayed and expressed as percentages of the activity obtained in a control group.

Malathion Carboxylesterase Titer and Malathion Toxicity - The total serum (S) and liver (L) malathion carboxylesterase activities measured in rats, in three strains of mice, and in five groups of mice pretreated with compound 3 (O,S,S-trimethyl phosphorothioate) are presented in Table 12, along with the corresponding LD_{50} values experimentally determined. The stochastic dependence of the variables (Table 12, columns 3 and 5) was investigated by linear regression analysis and a correlation coefficient (r) of 0.990 was obtained (95% confidence interval: $0.951 \leq r \leq 0.998$).

The equation of this regression line is:

$$LD_{50} = 84.7(S+L)-1220, \qquad (1)$$

where LD_{50} is the median lethal dosage in milligrams malathion per kilogram body weight; (S+L) is the sum of the total liver serum carboxylesterase activities in milligrams malathion hydrolyzed per minute per kilogram body weight; 84.7 is the slope of the regression line in minutes; and 1220 is the y-intercept in milligrams per kilogram body weight.

The response in C57B1 mice, deficient in serum carboxylesterase but not in liver carboxylesterase, suggested that the liver activity be weighted at twice the weight of the serum activity for the purpose of predicting malathion lethality. A regression line was obtained for the coordinate points $(S+2L)_n$, LD_{50n} (columns 4 and 5, Table 12). The correlation coefficient (r) for this set was calculated to be 0.995. The equation of this alternate regression line is:

$$LD_{50} = 42.4(S+2L)-429 \qquad (2)$$

TABLE 12. MALATHION CARBOXYLESTERASE ACTIVITY AND MALATHION TOXICITY IN RODENTS[a]

Animal group	1 Serum esterase (*S*) (mg MT/min/kg)	2 Liver esterase (*L*) (mg MT/min/kg)	3 *S*+*L*	4 *S*+2*L*	5 LD50 (mg MT/kg)
Sprague-Dawley	14.4	140	154	294	12,000
AKR mice	34.5	21.6	56.1	77.7	3,500
Swiss mice	30.6	27.5	58.1	85.6	3,200
C57Bl mice	10.3	23.5	33.8	57.3	2,600
Swiss mice + 5 mg/kg *O,S,S,*-TMP[b]	23.3	17.4	40.7	58.1	2,100
Swiss mice + 10 mg/kg *O,S,S*-TMP	22.2	16.6	38.8	55.4	1,590
Swiss mice + 20 mg/kg *O,S,S*-TMP	19.9	9.62	29.5	39.1	900
Swiss mice + 30 mg/kg *O,S,S*-TMP	11.0	7.7	18.7	26.4	425
Swiss mice + 78 mg/kg *O,S,S*-TMP	5.9	6.3	12.2	18.4	115

[a] Thirty animals in each group were used to determine the malathion (MT) LD50. In the cases of the *O,S,S*-TMP-treated animals, four from the original group of 34 animals were killed 4 hr after pretreatment to determine enzyme titers.

[b] *O,S,S*-Trimethyl phosphorodithioate.

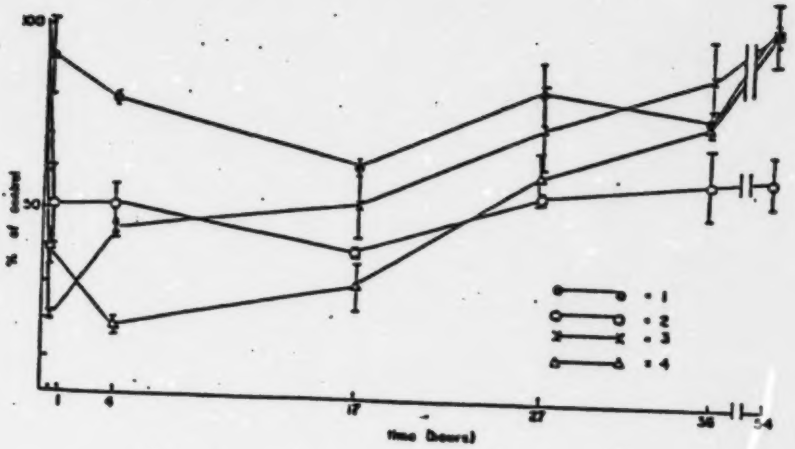


FIGURE 2

Variation of serum cholinesterase with time after dosing with compounds 1-4. Four groups of six rats received the test compounds at time zero, and blood samples were obtained from three of the six rats/group at various times after dosing. Residual cholinesterase activities in the sera were assayed. Results for each time point were averaged and expressed as percentages of activity measured in a control group. The mean ± SD obtained at each time point is shown in the figure.

where the terms and their units are analogous to those in Eq. (1). Although the correlation coefficients associated with the two lines do not differ significantly, the second line would appear to be preferable to the first as it provides a y-intercept closer to the expected value of zero. This equation was utilized to derive a prediction of the median lethal dosage of malathion in humans. Data on the activity of human liver were obtained in a study previously completed in the laboratory of Dr. Helmut Denk, University of Vienna. Malathion carboxylesterase activities were measured in 12 human livers showing normal histological appearance and the total activities were substituted into Eq. (2) above, (human serum has been reported to be devoid of malathion carboxylesterase[4], thus $S=0$). The predicted LD_{50} values are given in Table 13. The average LD_{50} value predicted was 3655 mg/kg (95% confidence limits: $2992 \leq x \leq 4318$ mg/kg).

A survey of the literature on human malathion poisonings revealed seven cases in which the ingested quantity was approximately known. The severity of the toxic signs (convulsions, coma, cyanosis, severe respiratory depression) and the protracted recovery periods associated with these cases indicated that the ingested doses were life-threatening. This clinical experience with the commercial malathion suggested that the life-threatening ingested dose is 500-1000 mg/kg, a value considerably lower than the value predicted from the capacity of human liver to detoxify the highly purified malathion. A probable explanation of the discrepancy is the fact that commercial preparations of malathion contain impurities that inactivate the detoxifying enzymes, the rodent data in Table 12 were obtained with malathion purified free of such impurities.

TABLE 13. MALATHION TOXICITY IN HUMANS PREDICTED FROM MEASUREMENTS OF HUMAN LIVER MALATHION CARBOXYLESTERASE ACTIVITY

Case	Liver esterase (mg MT/min/kg)	(S+2L)[a]	Estimated LD_{50}
mz	35	70	2536
fp	48	96	3683
ob	35.2	70.5	2557
ep	57	114	4400
rn	36.7	73.4	2680
jg	60	120	4654
fr	67.5	135	5290
gs	53.5	107	4104
rp	44.8	89.6	3367
sm	68	136	5339
gk	32	64	2282
rf	40.15	80.3	2972

[a] $S=0$ as human serum is devoid of malathion carboxylesterase activity. Data obtained in a survey of human liver carboxylesterase were substituted into the equation of the regression line relating the rodent toxicity measurements to S+2L. This equation is $LD_{50}=42.4\,(S+2L)-429$.

A soluble, partially purified preparation of human liver malathion carboxylesterase was obtained by detergent extraction and fractionation of whole liver homogenate[5]. This enzyme preparation was used to test four malathion impurities as inactivators of human liver malathion carboxylesterase activity. One of these compounds, isomalathion, effectively diminished enzyme activity at submicromolar concentrations, but the other compounds were ineffective, even at concentrations in the millimolar range (Table 14). Previous observations on rat liver carboxylesterase inactivation contrast with those reported in Table 14. In the rat, all four of the selected malathion impurities inactivated liver malathion carboxylesterase *in vitro* and *in vivo*.

TABLE 14. INACTIVATION OF HUMAN LIVER MALATHION CARBOXYLESTERASE BY IMPURITIES ISOLATED FROM TECHNICAL MALATHION[a].

Test compound	Result
Isomalathion	Concentration-dependent enzyme inactivation; IC_{50} = 0.6 μM
O,*O*,*S*-Trimethyl phosphorodithioate	No enzyme inactivation; highest concentration tested = 5 mM
O,*O*,*S*-Trimethyl phosphorothioate	No enzyme inactivation; highest concentration tested = 7.5 mM
O,*S*,*S*-Trimethyl phosphorodithioate	No enzyme inactivation; highest concentration tested = 7.5 mM

[a] Graded concentrations of the test compounds were incubated 1 hour at room temperature with 50 μl (70 μg protein) of the partially purified human liver malathion carboxylesterase, diluted with 0.25 ml of 0.1 M Tris-HCl, pH 7.5. At the end of the incubation period, 2.25 ml of the malathion carboxylesterase assay mixture was added, and the residual enzyme activity was determined colorimetrically (Talcott, 1979). In the case of isomalathion, the IC_{50} was estimated from a log-probability plot of percent enzyme inactivation vs isomalathion concentration.

Summary - Compounds previously identified as contaminants of technical malathion were found to diminish the activity of serum malathion carboxylesterase, liver malathion carboxylesterase, and serum cholinesterase *in vitro* and *in vivo*. The compounds tested were, in increasing order of potency, *O*,*O*,*S*-trimethyl phosphorodithioate, *O*,*O*,*S*-trimethyl phosphorothioate, *O*,*S*,*S*-trimethyl phosphorodithioate, and *O*,*S*-dimethyl S-(1,2-dicarboethoxy) ethyl phosphorodithioate. *In vivo*, the diminutions of serum esterase activity caused by these compounds were transient, with the exception of the diminutions caused by O,O,S-trimethyl phosphorothioate, which persisted for at least 54 hr. The most potent of these esterase inactivators, also known as isomalathion, is of particular interest as a possible contributor to an epidemic of malathion poisonings among malaria workers in Pakistan.

The titer of malathion carboxylesterase in liver and sera of rats, three strains of mice, and five groups of pretreated mice was correlated with the malathion LD_{50} values measured in these groups. The equation of a regression line relating enzyme titer to toxicity was used to predict the median lethal dosage of purified malathion to humans. The mean value, 3655 mg/kg, was compared with dosages ingested in actual cases of human malathion poisonings. A discrepancy was noted between the LD_{50} value predicted in this study and the life-threatening doses of commercial malathion reported in the clinical literature. The unexpectedly high toxicity of commercial malathion may be related to its content of esterase-inactivating impurities.

A soluble stable and partially purified preparation of human liver malathion carboxylesterase was obtained by detergent extraction and fractionation of whole liver homogenate. The activity of this fraction was susceptible to concentration-dependent inactivation by isomalathion but was resistant to three other impurities isolated from technical malathion. The potency of isomalathion as a malathion carboxylesterase inactivator supports the possibility that this compound may have contributed to the malathion poisonings in Pakistan.

3. Properties of Rat Malathion Carboxylesterase.

Introduction - In continuing our investigations on the effect of impurities on the toxicological properties of technical organophosphorus insecticides, we have examined two distinct esterase fractions isolated from rat liver microsomes[5] for enzymatic hydrolysis of [methoxy-^{14}C]malathion with special attention given to product analysis. The effect of O,S,S-trimethyl phosphorodithioate and isomalathion as inhibitors of these esterases also was examined.

Isolation and Characterization of Rat Liver Carboxylesterase - Specific activities for the hydrolysis of malathion to the monoacid by the various fractions isolated on route to the final partially purified fractions, i.e., esterase fraction A and esterase fraction B, are presented in Table 15. The results reveal that fraction A and fraction B are 13- and 18-fold more effective, respectively, in hydrolyzing malathion than the microsomal fraction. The two fractions could not be distinguished kinetically, but the esterase fraction A contained at least one electrophoretic species not present in esterase fraction B (see Table 16).

TABLE 15. ENZYMATIC ACTIVITY OF STEPWISE FRACTIONS OBTAINED IN THE PURIFICATION OF RAT LIVER MALATHION CARBOXYLESTERASE

Fraction	Specific activity (nmol/min/mg protein)	Total activity[a] (nmol/min)
Microsomes	44.6	70,000
Triton X-100 supernatant	158.0	58,285
Post-acetone supernatant	164.0	15,215
Hydroxylapatite chromatography fractions		
Esterase fraction A	586.0	9,000
Esterase fraction B	814.0	11,142

[a] Activity present in indicated fraction obtained from 136 g rat liver.

TABLE 16. PROPERTIES OF ESTERASE FRACTION A AND ESTERASE FRACTION B OBTAINED FROM RAT LIVER

	Fraction	
Property	Esterase fraction A	Esterase fraction B
V_{max} (μmole malathion hydrolyzed/min/mg protein)	1.13	1.68
K_m (mM) for malathion	0.117 ± 0.017	0.156 ± 0.026
Molecular weight	50,000 - 60,000	110,000 - 130,000
Electrophoretic bands	0.18 (major); 0.24, 0.38 (minor)	0.15 (major); 0.24 (minor)

Data showing the identification and amounts of degradation products isolated after incubation of [methoxy-^{14}C]malathion with esterase fraction A, esterase fraction B, and crude liver homogenate are given in Table 17. The principal reaction mediated by the crude homogenate or purified esterases was the hydrolysis of the carboethoxy moiety, leading to either α- or β-malathion monoacid with minor formation of other products. The selectivity of the purified esterases for the two different carboethoxy moieties is of interest. While the crude liver homogenate hydrolyzed malathion to α- and β-monoacid in a ratio of 1:1.8 after 30 min of incubation period, the α/β ratio for esterase fraction A was 3:2 and that for esterase fraction B was 1:5. Thus, fraction A produced larger amounts of α- than β-monoacid and fraction B produced much larger amounts of β- than α-monoacid. The ratio of monoacids generated by the crude homogenate was in general agreement with the sum of each monoacid produced by the two different esterase fractions.

Inhibition of Carboxylesterase - Of the various impurities present in technical malathion, O,S,S-trimethyl phosphorodithioate and the S-methyl isomeride of malathion (isomalathion) are known to be the strongest potentiators of the mammalian toxicity of malathion. These two compounds were selected for examination as inhibitors of the purified carboxylesterases and also crude rat liver homogenate. Bimolecular rate constants (k_i) for the inhibition of the carboxylesterases are given in Table 18. According to the data, isomalathion was 6- to 12-fold more effective in inhibiting the three esterases than O,S,S-trimethyl phosphorodithioate. Isomalathion, with k_i values in the neighborhood of $2.0 \times 10^5 \underline{M}^{-1}\text{min}^{-1}$ proved to be a highly potent inhibitor of rat liver carboxylesterase. In fact, it is a stronger inhibitor of rat liver carboxylesterase than it is of rat serum cholinesterase.

Data showing the effect of O,S,S-trimethyl phosphorodithioate and isomalathion on the degradation of [methoxy-^{14}C]malathion by purified and crude rat liver carboxylesterase are presented in Table 19. In general, the data are in agreement with the k_i values and reveal greater antiesterase activity for isomalathion compared to the trimethyl ester. Although O,S,S-trimethyl phosphorodithioate and isomalathion were both effective in reducing the amounts of α- and β-monoacid generated from malathion by the carboxylesterases, they appeared to affect the enzyme preparations in different ways. For example, in the presence of 5 μM O,S,S-trimethyl phosphorodithioate the reduction in α-monoacid generated from malathion by esterase fraction A was about 4-fold compared to about a 1.5-fold reduction in the generation of β-monoacid. Esterase fraction B was affected similarly at 5 μM of the trimethyl ester, the amount of α-monoacid being reduced 4.4-fold and β-monoacid being reduced 1.6-fold. Thus, the reaction leading to α-monoacid was more strongly inhibited by O,S,S-trimethyl phosphorodithioate than the reaction leading to the β-monoacid. In contrast, in all three enzyme fractions the reactions leading to either α- or β-monoacid were inhibited to approximately an equal degree by isomalathion. In terms of spectrum and magnitude of inhibitory activity, isomalathion was substantially superior to O,S,S-trimethyl phosphorodithioate as an inhibitor of rat liver malathion carboxylesterase.

TABLE 17. NATURE AND AMOUNTS OF PRODUCTS FORMED UPON DEGRADATION OF [METHOXY-^{14}C]MALATHION BY CRUDE AND PURIFIED RAT LIVER CARBOXYLESTERASE AT 37°C AND pH 7.4[a]

Product	Relative amounts of products obtained (%)						
	Esterase fraction A		Esterase fraction B		Crude homogenate		Control (no enzyme)
	5 min	30 min	5 min	30 min	5 min	30 min	30 min
Malathion	89.4	57.2	89.3	70.4	84.2	45.1	98.8
α-Monoacid	5.5	23.3	0.7	4.2	5.0	17.9	0
β-Monoacid	3.1	15.2	5.5	21.7	8.0	32.2	0
Origin	0.1	0.1	0.1	0.1	0.1	0.2	0
Unextractables	0.1	0.3	0.1	0.5	0.2	0.4	0
Total radioactivity recovered	98.2	96.1	95.7	96.9	97.5	95.8	98.8

[a] Six units of enzyme, 30 min incubation.

TABLE 18. BIMOLECULAR RATE CONSTANTS FOR THE INHIBITION OF CRUDE AND PURIFIED RAT LIVER CARBOXYLESTERASE BY O,S,S-TRIMETHYL PHOSPHORODITHIOATE AND ISOMALATHION AT 37°C and pH 7.4

Compound	k_i ($\underline{M}^{-1}$min^{-1})		
	Esterase fraction A	Esterase fraction B	Crude homogenate
$(CH_3S)_2P(O)OCH_3$	1.6×10^4	2.3×10^4	2.6×10^4
Isomalathion	2.0×10^5	1.7×10^5	1.5×10^5

TABLE 19. EFFECT OF O,S,S-TRIMETHYL PHOSPHORODITHIOATE AND ISOMALATHION ON THE DEGRADATION OF [METHOXY-^{14}C]MALATHION BY PURIFIED AND CRUDE RAT LIVER CARBOXYLESTERASE[a]

Product	Relative amounts of products obtained (%)									
	Esterase fraction A			Esterase fraction B			Crude homogenate			
		$(CH_3S)_2P(O)OCH_3$			$(CH_3S)_2P(O)OCH_3$			$(CH_3S)_2P(O)OCH_3$		
	Control[b]	5 µM	10µM	Control[b]	5 µM	10µM	Control[b]	5 µM	10µM	500µM
Malathion	12.9	62.8	83.1	33.4	60.8	84.1	5.1	30.7	43.6	97.4
α-Monoacid	55.9	14.4	5.8	10.1	2.3	1.0	36.0	9.3	8.1	0.5
β-Monoacid	21.5	14.3	7.1	46.0	28.3	13.4	52.3	56.1	44.0	0.7
Origin	0.3	0.3	0.2	0.3	0.4	0.1	0.3	0.3	-	0.1
Unextractables	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.1	0.3
Total radio-activity recovered	90.9	92.1	96.5	90.1	92.1	98.9	94.0	96.7	95.8	99.0
		Isomalathion			Isomalathion			Isomalathion		
	Control	0.1µM	5 µM	Control	0.1µM	5 µM	Control	0.1µM	5 µM	
Malathion	12.9	46.2	89.3	33.4	53.3	90.4	5.1	37.4	90.5	
α-Monoacid	55.9	31.4	2.6	10.1	7.6	1.3	36.0	23.7	2.8	
β-Monoacid	21.5	16.1	3.9	46.0	34.2	4.9	52.3	34.1	5.2	
Origin	0.3	0.2	0.3	0.3	0.2	0.3	0.3	0.3	0.2	
Unextractables	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.2	0.3	
Total radio-activity recovered	90.9	94.2	96.4	90.1	95.6	97.2	96.7	95.7	99.0	

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[a] Twenty-four units of enzyme, 30 min incubation, pH 7.4 at 37°C.

[b] No inhibitor.

Rat Serum Carboxylesterase - The degradation of [methoxy-^{14}C]malathion catalyzed by rat serum carboxylesterase and the antiesterase properties of O,S,S-trimethyl phosphorodithioate and isomalathion against this enzyme preparation also were studied. Results are presented in Table 20. In contrast to crude rat liver carboxylesterase, which generated almost 2-fold more β-monoacid from [methoxy-^{14}C]malathion than the α-monoacid, serum carboxylesterase formed approximately equal quantities of α-monoacid and β-monoacid. As in the case of the carboxylesterases obtained from liver, isomalathion was substantially more effective than O,S,S-trimethyl phosphorodithioate in inhibiting serum carboxylesterase. The bimolecular inhibition constants calculated from the data in Table 20 were (k_i, $M^{-1}min^{-1}$) 1.3 x 10^3 for O,S,S-trimethyl phosphorodithioate and 1.4 x 10^5 for isomalathion. Therefore, O,S,S-trimethyl phosphorodithioate appeared to be less effective in inhibiting serum carboxylesterase than rat liver carboxylesterase and isomalathion inhibited both enzymes to approximately an equal degree.

Summary - Two malathion carboxylesterase fractions, designated as esterase fraction A and esterase fraction B, which hydrolyze malathion were purified 13- and 18-fold, respectively, from rat liver microsomes. The two enzymes could not be distinguished kinetically from each other, but the fraction A contained at least one electrophoretic species not present in the fraction B. The molecular weight of esterase fraction A was estimated as 50,000-60,000, but the molecular weight of esterase fraction B was about twice this value. Incubation of [methoxy-^{14}C]malathion with fraction A or fraction B resulted in a mixture of malathion α- and β-monoacids but the composition of the mixture produced by fraction A (α/β ratio = 1.5) differed from that produced by fraction B (α/β ratio = 0.2), indicating the presence of multiple species of carboxylesterases in mammalian liver microsomes. Isomalathion was substantially more potent as an inhibitor of both rat liver and rat serum malathion carboxylesterases than O,S,S-trimethyl phosphorodithioate. Isomalathion appeared to be equipotent in inhibiting the rat liver carboxylesterase catalyzed reactions leading to either α- or β-monoacid. O,S,S-Trimethyl phosphorodithioate, on the other hand, preferentially diminished the reactions leading to α-monoacid. In contrast, the rat serum carboxylesterase catalyzed reactions leading to either α- or β-monoacid were inhibited to approximately an equal degree by either isomalathion or O,S,S-trimethyl phosphorodithioate.

The overall potency of isomalathion in inhibiting both rat liver and rat serum carboxylesterase indicates that it, among the various impurities present in technical malathion, is the major contributor to malathion potentiation.

4. References.

1. Frawley, J.P., H.N. Fuyat, E.C. Hagan, J.R. Blake, O.C. Fitzhugh, J. Pharmacol. Exp. Ther. 121, 96 (1957).
2. Pellegrini, G., R. Santi, J. Agric. Food Chem. 20, 944 (1972).
3. Dauterman, W.C., Bull. Wld. Hlth. Org. 44, 133 (1971).
4. Main, A.R. and P.E. Braid, Biochem. J. 84, 255-63 (1962).
5. Haugen, D.A. and J.W. Suttie, Biol. Chem. 249, 2717-22 (1974).

TABLE 20. EFFECT OF O,S,S-TRIMETHYL PHOSPHORODITHIOATE AND ISOMALATHION ON THE DEGRADATION OF [METHOXY-^{14}C]MALATHION BY RAT SERUM CARBOXYLESTERASE[a]

Inhibitor	Concentration of inhibitor (µM)	Relative amounts of products obtained (%)		
		Malathion	α-Monoacid	β-Monoacid
$(CH_3S)_2P(O)OCH_3$	0 (control)[b]	22.6	40.9	34.9
	10	57.4	19.1	22.3
	15	61.9	14.9	21.8
	20	66.4	16.3	16.1
	25	72.6	13.2	13.2
	40	93.8	2.4	2.6
Isomalathion				
	0 (control)[b]	22.6	40.9	34.9
	0.25	66.8	16.3	15.6
	0.5	88.4	5.3	5.4
	1.0	98.6	0.1	0.3
	1.5	98.3	0.1	0.4

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[a] Six units of enzyme (5 µl serum), 30 min incubation, pH 7.4 at 37°C.

[b] No inhibitor.

F. DELAYED TOXICITY OF TRIALKYL PHOSPHOROTHIOATES

1. Delayed Toxicity of *O,O,S*-Trimethyl Phosphorothioate.

Introduction - In the preceding section, we reported the presence of O,O,S-trimethyl phosphorothioate as an impurity in technical malathion, along with other sulfur containing trimethyl esters. The acute oral LD_{50} of this compound was reported as 260 mg/kg based on 24-48 hr mortality. In continuing studies on the potentiating and toxicological properties of these impurities in rats and mice, we discovered that O,O,S-trimethyl phosphorothioate was a far more hazardous material than estimated from 48 hour mortality data. This section is concerned with delayed toxic effects which are observed in rodents after treatment with simple trialkyl phosphorothioates and phosphorodithioates.

Delayed Toxicity of *O,O,S*-Trimethyl Phosphorothioate (I) to Rats - Compared to intoxication by an anticholinesterase organophosphorus insecticide, rats treated orally with I required substantially longer holding periods after treatment before mortality occurred. This is evident in the data in Table 1 which reveals holding periods as long as 22 days before death occurred. The estimated LD_{50} of I was 15-20 mg/kg, making this compound approximately equal to parathion in rat toxicity. Because of the extended period of time for death to occur, the term delayed toxicity is used to describe intoxication brought about by this compound.

At the higher dosages, e.g., 60-200 mg/kg, diarrhea, excessive urination, bleeding from the mouth, nose and ear were common among the treated animals (Table 2). These signs of poisoning were slight at 30 mg/kg and in most cases were absent at 15 mg/kg. Although not immediately obvious from casual observation, the prevailing symptom of intoxication was weight loss. In many cases, weight loss was severe, with rats losing a third to almost one-half of their original weight 4-5 days following treatment. All of the rats treated at the low dose of 15 mg/kg appeared to be normal in physical appearance except for weight loss.

Several rats orally treated with I at different doses were dissected for examination of their internal organs. At the doses of either 60 or 200 mg/kg, internal hemorrhaging was observed in the lungs, liver, intestine and stomach. The stomach and intestine were virtually empty of food at 4 days after treatment of the toxicant. The levels of white and yellow fat were extremely low.

Since the initial studies in rats were conducted after oral treatment, the effect of intraperitoneal administration also was examined and the same signs of delayed toxicity also were observed.

To determine whether delayed toxicity caused by I was cumulative, the toxicant was orally administered daily to 5 rats for 12 days at the sublethal dose of 6 mg/kg (about 1/3 of LD_{50} value). At the end of 12 days (total dose of 72 mg/kg compound) these rats did not reveal any

TABLE 1. TIME OF DEATH OF RATS TREATED ORALLY WITH DIFFERENT AMOUNTS OF *O*,*O*,*S*-TRIMETHYL PHOSPHOROTHIOATE (I)

Dose of I (mg/kg)	No. of rats treated	Time (days) of occurrence of death									Total rats killed	% killed
		1	2	3	4	5	6	7	8	9-25		
300	4		4								4	100
200	4		2	2							4	100
80	4				2		1		1		4	100
60	18			2	4	4	4	3	1		18	100
40	4				2	-	1		1		4	100
30	4				2	1	1				4	100
20	4						1		1	1[a]	3	75
15	4				1					1[b]	2	50
10	4										0	0
0	6										0	0

[a] Died on 17th day; [b] Died on 22nd day.

TABLE 2. TOXICOLOGICAL SYMPTOMS IN RATS TREATED WITH O,O,S-TRIMETHYL PHOSPHOROTHIOATE

Dose (mg/kg)	Rat	Symptoms* (a, b and c) after treatment (days)						
		1	2	3	4	5	6	9
		a b c	a b c	a b c	a b c	a b c	a b c	a b c
200	Rat I	10 ++ +	17 ++ +	18 (died)				
	Rat II	8 ++ +	11 (died)					
	Rat III	15 + +	21 ++ -	24 (died)				
	Rat IV	10 + +	14 (died)					
30	Rat I	15 - +	24 + +	31 - -	37 (died)			
	Rat II	16 - +	23 - -	29 - -	37 (died)			
	Rat III	14 - ±	23 - -	30 - -	35 - -	40 - -	46 (died)	
	Rat IV	12 - +	22 - +	27 - -	32 - -	35 (died)		
15	Rat I	8 - -	15 - -	11 - -	6 - -	5 - -	+1 - -	+7 - -
	Rat II	1 - -	3 - -	3 - -	+1 - -	+2 - -	+7 - -	+3 - -
	Rat III	10 - -	14 - -	10 - -	8 - -	+2 - -	+6 - -	+2 - -
	Rat IV	6 + -	17 - -	25 - -	30 (died)			
0 (corn oil only)	Rat I	+4 - -	+10 - -	+16 - -	+16 - -	+21 - -	+25 - -	+37 - -
	Rat II	+9 - -	+16 - -	+21 - -	+22 - -	+30 - -	+35 - -	+55 - -

*

a: % weight loss from initial weight.

b: diarrhea or urination: ++, heavy; +, observed; -, not observed.

c: bleeding from mouth, nose or ear: ++, heavy; +, observed; -, not observed.

poisoning signs except for suppression of weight increase. On the 2nd to 4th day, weight loss was maximum (11-16% compared to initial weight) and thereafter the weight of the rats started to increase, even though the toxicant was administered each day until the 12th day. The animals were observed for another 8 days after termination of the treatment and no weight loss nor poisoning signs were observed.

Delayed Toxicity of *O,O,S*-Trimethyl Phosphorothioate (I) to Mice - Studies on the delayed toxicity of I also were examined in mice. As shown in Table 3, in general, the same kinds of symptoms appeared in mice as appeared in rats; however, the time pattern for the occurrence of death for the two animals was different with mice exhibiting a much narrower time frame. Most of the mice died during the 2nd-6th days after oral treatment of 75 to 200 mg/kg I, but this compound appeared to be significantly less toxic to mice than to rats. The principal signs of poisoning in mice treated with 75 to 200 mg/kg I were weight loss and lacrimation. Diarrhea, excessive urination and bleeding, which were typical poisoning signs with the rat were not observed with the mouse. The eyes of mice treated with the toxicant contained large amounts of mucous and severely affected mice were unable to open their eyes. This eye abnormality was most severe during the first 2 days after treatment and then gradually disappeared in animals which survived. Lacrimation was not observed in the rat.

Delayed Toxicity of *O,O,S*-Triethyl Phosphorothioate (II) and Related Esters to Rats - O,O,S-Triethyl phosphorothioate (II) was examined for delayed toxicity since this material may be present or generated in technical diethyl phosphorothioate insecticides, e.g., parathion. Rat mortality data at different doses are given in Table 4. While the same kinds of intoxication symptoms were observed with this ester, i.e., diarrhea, excessive urination, etc., the time pattern of the occurrence of death was somewhat different than that for the trimethyl analog. At the higher doses (85-110 mg/kg), death occurred within 24 hr after treatment. However, at the lower dose of 50 mg/kg, death occurred on the 4th and 8th day. The LD_{50} value for the triethyl ester after 48 hr was estimated to be about 80 mg/kg, while a value of 45-90 mg/kg was estimated after 8 days. Thus, relatively little difference in LD_{50} was observed between the two time periods.

The toxicities of O,O,S-trimethyl (VI) and O,S,S-trimethyl phosphorodithioate (VII), two impurities present in technical malathion also were examined. Our preliminary results showed that VI and VII did not elicit delayed toxic effects in rats. However, re-examination of these compounds after further purification by TLC and column chromatography showed VII to produce the same kinds of intoxication symptoms of delayed toxicity following oral treatment of rats as those caused by I. The estimated rat oral LD_{50} of VII was 40-70 mg/kg.

Effect on Food and Water Uptake - Since toxicological studies revealed severe weight loss in rats treated with the toxicant, the effect of the toxicant on food and water uptake and weight change in rats was investigated. Rats were treated with 60 mg/kg I and the amounts of food and water uptake

TABLE 3. TIME OF DEATH OF MICE TREATED ORALLY WITH DIFFERENT AMOUNTS OF O,O,S-TRIMETHYL PHOSPHOROTHIOATE (I).

Dose of I (mg/kg)	No. of mice treated	Time (days) of occurrence of death 1	2	3	4	5	6	7	8-15	Total mice killed	% killed
200	5	1	3	1						5	100
150	5		1	1						2	40
125	5		1				1			2	40
100	5		2							2	40
75	5			1			1			2	40
50	5									0	0

TABLE 4. TIME OF DEATH OF RATS TREATED ORALLY WITH DIFFERENT AMOUNTS OF O,O,S-TRIETHYL PHOSPHOROTHIOATE (II).

Dose of II (mg/kg)	No. of rats treated	Time (days) of occurrence of death 1	2	3	4	5	6	7	8	9-25	Total rats killed	% killed
110	4	3					1				4	100
100	5	4									4	80
85	4	1	1								2	50
75	4		2								2	50
50	4				1				1		2	50
30	4										0	0

and weight changes were determined every 24 hr for 4 days. The results (Table 5) show that the rats treated with the toxicant stopped eating and drinking within 24 hours and intake of neither food nor water was observed until death occurred. The amounts of urine and feces collected from treated rats were significantly smaller than from untreated animals. The pH ranges of the urine from the treated animals were lower (5.8~6.3) than those of the nontreated animals (6.9~8.6).

Effect of Administration of Nutrient Solution - Rats treated with 60 mg/kg I were injected with 2.0 ml of nutrient solution (hyperalimentation) twice a day for 5 days to determine if mortality could be delayed or blocked. Injection of nutrient solution in two rats orally dosed with I did not cause any delay or block of the death of animals. This finding, coupled with the observation that two control rats given only nutrient solution managed to survive the test period (10 days), suggests that the death of the animals following treatment of I is not attributable simply to starvation. The two control rats kept without any water, food, and nutrient solution died on the 4th and 7th day, respectively.

Effect of Atropine - Rats treated with 60 mg/kg I were subsequently treated with different doses of atropine to determine if the intoxicating effects could be reduced or eliminated. The results (Table 6) show that atropine at the doses of 20-120 mg/kg did not block or reduce poisoning effects, though death of some rats treated with 60 mg/kg of the toxicant and 60 mg/kg of atropine was blocked in spite of heavy poisoning symptoms. Overall, the results suggest a noncholinergic mechanism of intoxication for I.

Inhibition of Rat Serum Carboxylesterase and Cholinesterase - Previous studies on the mode of potentiating action of I and other malathion potentiators showed that both serum carboxylesterase and cholinesterase were significantly inhibited for extended periods after a single oral dose of the trimethyl phosphorothioate. Owing to the likelihood that *O,O,S*-trimethyl phosphorothioate and related esters are inhibitors of esterases in general, additional studies on rat serum carboxylesterase and cholinesterase were carried out to determine whether any relationship was apparent between the inhibition of these enzymes and delayed toxic activity. Results are provided in Table 7 which show the levels of serum carboxylesterase and cholinesterase activity present in blood samples taken from rats at different time intervals following single oral doses of different amounts of either I or II. Both serum carboxylesterase and cholinesterase were inhibited to approximately equal degrees after treatment with each compound although inhibition of carboxylesterase appeared to be greater during the early stages after treatment (4 and 20 hr). In general, spontaneous recovery of both carboxylesterase and cholinesterase activity was slow after inhibition by either compound and, further, most of the animals whose enzymatic activity remained inhibited for extended periods eventually died.

Antagonism of the Toxicity of O,O,S-Trimethyl Phosphorothioate (I) by the O,O,O-Trimethyl Isomer (III) - During the evaluation of the toxicity of *O,O,S*-trimethyl phosphorothioate to rats, it was observed that the sample

TABLE 5. EFFECT OF O,O,S-TRIMETHYL PHOSPHOROTHIOATE (I) ADMINISTERED ORALLY AT 60 mg/kg ON FOOD AND WATER UPTAKE AND THE WEIGHT CHANGE IN RATS

	Rat no.	Food (a) and water (b) uptakes and weight changes* (c) after treatment											
		0-24 hrs			24-48 hrs			48-72 hrs			72-96 hrs		
		a (g)	b (ml)	c (%)	a (g)	b (ml)	c (%)	a (g)	b (ml)	c (%)	a (g)	b (ml)	c (%)
Treated (60 mg/kg)	1	2	2	-14	0	0	-22	0	0	-25	0.6	0.5	-28
	2	3	4	-10	0	0	-16	0	0	-29	0	0	-29
Non-treated	1	13	28	+9	14	25	+13	15	23	+20	16	24	+20
	2	12	18	+7	14	19	+11	14	17	+15	14	20	+15
Control (without food and water)	1			-12			-20			-26			-31
	2			-12			-22			-29			-36 (died)

*% weight changes from initial weight at the end of each period.

TABLE 6. EFFECT OF DIFFERENT DOSES OF ATROPINE ON THE TOXICITY OF 60 mg/kg O,O,S-TRIMETHYL PHOSPHOROTHIOATE (I) ADMINISTERED ORALLY TO RATS

Dose of I (mg/kg)	Amount of atropine administered (mg/kg)	No. of animals	Time (days) of occurrence of death								Total rats killed	% killed
			1	2	3	4	5	6	7	8-25		
60	10	2				1	1				2	100
60	20	4			1	1	1			1	4	100
60	60	8				4	1				5	62
60	120	2			1	1					2	100
-	20	2									0	0
-	60	2									0	0

TABLE 7. IN VIVO INHIBITION OF RAT SERUM CARBOXYLESTERASE AND CHOLINESTERASE AT DIFFERENT TIME INTERVALS FOLLOWING ORAL ADMINISTRATION OF DIFFERENT DOSES OF O,O,S-TRIMETHYL (I) AND O,O,S-TRIETHYL PHOSPHOROTHIOATE (II).

Compound	Dose (mg/kg)	Rat no.	Percent control activity						
			4 h	20 h	2 days	3 days	6 days	7 days	14 days
Rat Serum Carboxylesterase									
I	15	1	38.7		73.1		55.6		100.0
		2	80.6		85.7		61.1		32.2[a]
		3	58.1		75.6		66.7		100.0
		4	48.4		70.6[a]				
	40	1	45.7		-			43.7[a]	
	60	1	12.7	18.2		33.3[a]			
		2	13.0	27.0		43.5[a]			
II	50	1	7.7		70.5		37.8		94.4
		2	7.7		85.7		100.0		100.0
		3	12.9		45.9[a]				
		4	3.2		40.3		38.9[a]		
	85	1	4.5		27.7		75.0		100.0
		2	3.9[a]						
		3	6.5		50.4		77.8		100.0
		4	5.8		4.5[a]				
Rat Serum Cholinesterase									
I	15	1	63.2		59.3		66.7		100.0
		2	61.1		48.2		51.9		68.4[a]
		3	68.4		56.3		63.0		100.0
		4	73.7		60.7[a]				
	60	1	43.8			75.0[a]			
		2	42.7			80.0[a]			
	50	1	29.5		55.6		44.4		100.0
		2	29.5		63.0		63.0		100.0
		3	31.6		49.6[a]				
		4	26.3		49.6		37.0[a]		
	85	1	13.7		32.6		40.7		82.1
		2	13.7[a]						
		3	23.2		40.7		51.9		100.0
		4	21.1		19.3[a]				

---

[a] This animal eventually died.

of this compound prepared by the method utilizing the reaction between trimethyl phosphite and methylsulfenyl chloride and purified by vacuum distillation could be administered at doses up to 200 mg/kg without mortality and obvious signs of intoxication, i.e., diarrhea, excessive urination, and bleeding. The low toxicity of this material contrasted markedly to the toxicity of a sample of *O*,*O*,*S*-trimethyl phosphorothioate (I) previously prepared by reacting *O*,*O*-dimethyl phosphorothioic acid with dimethyl sulfate and purified by column chromatography. Although the NMR spectra of these two samples were virtually identical, the low toxicity of the material from the second preparation (referred to as Ia) suggested that it was contaminated with an impurity which was antagonizing its toxicity.

TLC analysis [solvent system:hexanes-ether (1:1)] of Ia showed the presence of an impurity (Rf:0.64) which gave a pink coloration with DBQ. The Rf value corresponded to that of *O*,*O*,*O*-trimethyl phosphorothioate (III), the P=S isomer of *O*,*O*,*S*-trimethyl phosphorothioate. Therefore, a 500 mg sample of Ia was subjected to column chromatographic purification using 25 g of silica gel and 400 ml of hexanes-ether (3:2) as the eluting solvent.

An impurity was isolated which was identified as *O*,*O*,*O*-trimethyl phosphorothioate (III). The amount of III in the vacuum distilled sample of Ia was 2.5∿3.2%, as estimated by GC.

Following the identification of the impurity, the remaining Ia was purified by column chromatography. The purified sample (I) was then adulterated with small increments of *O*,*O*,*O*-trimethyl phosphorothioate (III) and the rat toxicities of the mixtures were evaluated. The results of this study are presented in Table 8. These data show that the presence of the thiono isomer (III) blocks the mortality and poisoning symptomology otherwise associated with administration of the thiolate (I) alone. The external appearance and behavior of the treated rats was not visibly different from those of the controls. At doses of either 60 or 200 mg/kg of the toxicant, 1% (w/w) of the antagonist was sufficient to protect the rats from poisoning. Higher doses of I were not utilized in this study since the distinction between delayed and acute toxicity becomes obscure. In the latter case, the poisoning signs were also characteristically cholinergic. For rats treated with I at 60 mg/kg, decreasing the level of III below 1% appeared to give partial protection and at 0.5%, half of the treated animals recovered. Similar antagonism effects also were noted in mice. Administration of 200 mg/kg I containing 3% III to mice prevented death, although some signs of poisoning were observed.

Administration of 3% of III 24 and 48 hours after treatment with I failed to protect rats from poisoning effects or mortality. However, pretreatment with III either 4 or 24 hours before administration of I succeeded in blocking mortality and poisoning signs. Administration of I containing 3% *O*,*O*,*O*-triethyl phosphorothioate (IV), the triethyl analog of III, did not cause any antagonism of the delayed toxicity of I. Further, administration of I containing 3% trimethyl phosphate (V), the oxon of III, also failed to antagonize the toxicity of I.

TABLE 8. EFFECT OF ORALLY ADMINISTERED O,O,O-TRIMETHYL PHOSPHOROTHIOATE (III), O,O,O-TRIETHYL PHOSPHOROTHIOATE (IV) AND O,O,O-TRIMETHYL PHOSPHATE (V) ON THE TOXICITY OF ORALLY ADMINISTERED O,O,S-TRIMETHYL PHOSPHOROTHIOATE (I) TO RATS

Dose of I (mg/kg)	Amount of III, IV or V added, %		No. of rats treated	Time (days) of occurrence of death								Total rats killed	% killed
				1	2	3	4	5	6	7	8-25		
200			5		2	3						5	100
200	III	0.1	4	1	3							4	100
200	III	0.5	4		4							4	100
200	III	1.0	6									0	0
200	III	3.0	4									0	0
60			6			1		4	1			6	100
60	III	0.1	4				1	1	1			3	75
60	III	0.5	7				2		2			4	57
60	III	1.0	6									0	0
60	III	3.0	8									0	0
60	III	3.0[a]	4		1				2	1		4	100
60	III	3.0[b]	2				1	1				2	100
60	III[c]	20.0	2									0	0
60	III[d]	20.0	4									0	0
60	IV	3.0	4			1	3					4	100
60	V	1.0	2					2				2	100
60	V	3.0	4				1	2			1[e]	4	100

[a] Dosed orally 24 h after treatment with I; [b] Dosed orally 48 hr after treatment with I; [c] Pretreated orally 4 h before treatment with I; [d] Pretreated orally 24 h before treatment with I; [e] Died on ninth day.

TABLE 9. *IN VIVO* INHIBITION OF RAT SERUM CARBOXYLESTERASE AND CHOLINESTERASE AT DIFFERENT TIME INTERVALS FOLLOWING ORAL ADMINISTRATION OF *O*,*O*,*S*-TRIMETHYL PHOSPHOROTHIOATE (I) CONTAINING INCREASING INCREMENTS OF *O*,*O*,*O*-TRIMETHYL PHOSPHOROTHIOATE (III).

Dose of I (mg/kg)	Amount of III added (%)	Rat no.	Percent control activity		
			4 h	1 day	6 days
Rat Serum Carboxylesterase					
200	0.1	1		10.9(2d)[a]	
		2		11.4(2d)	
	0.5	1		8.6(2d)	
		2		8.6(2d)	
	1.0	1	8.3	8.3	100
		2	0.9	5.0	80
60	0.1	1		8.6(4d)	
		2		14.3(5d)	
	0.5	1		9.1(4d)	
		2		14.3(4d)	
	1.0	1	34.5	20.7	100
		2	13.8	31.0	100
Rat Serum Cholinesterase					
200	0.1	1		29.4(2d)	
		2		11.8(2d)	
	0.5	1		25.9(2d)	
		2		23.5(2d)	
	1.0	1	10.7	29.4	100
		2	12.5	39.9	100
60	0.1	1		36.5(4d)	
		2		33.3(5d)	
	0.5	1		32.9(4d)	
		2		32.9(4d)	
	1.0	1	60.0	35.0	100
		2	40.0	40.0	100

[a] Day of death in parenthesis.

The effect of III on the *in vivo* inhibition of rat serum carboxylesterase and cholinesterase by I was examined. O,O,S-Trimethyl phosphorothioate adulterated with 0.1-1.0% III was administered orally to rats and enzymatic activity was determined at 3 different time intervals (Table 9). The data presented earlier in Table 7 showed that I caused significant and prolonged inhibition of rat serum carboxylesterase and cholinesterase following administration of a single oral dose of 15, 40, or 60 mg/kg. While the significance of this extended inhibition is not known, it is noteworthy that when III was co-administered at 1%, total recovery of both enzymes was observed 6 days after treatment at 60 and 200 mg/kg although the enzymes were initially strongly inhibited. These animals also survived the poisoning effects of I.

Summary - Simple trialkyl phosphorothioates and dithioates, esters which were generally believed to be nontoxic compounds, can be highly toxic materials of an insidious nature. O,O,S-Trimethyl phosphorothioate, an impurity present in several technical organophosphorus insecticides, administered orally to rats caused delayed mortality at single doses as low as 15 mg/kg, with death occurring 4-22 days following treatment. Delayed toxic signs also were observed in mice but mice generally were less sensitive than rats. O,O,S-Triethyl phosphorothioate and O,S,S-trimethyl phosphorodithioate induced the same signs of intoxication at slightly higher doses. Rats treated with O,O,S-trimethyl phosphorothioate refused food and water within 24 hr after treatment until the time of death. Neither injection of nutrient solution nor atropine served to reduce or block intoxication. However, the isomeric O,O,O-trimethyl phosphorothioate was a potent antagonist of the toxicity of O,O,S-trimethyl phosphorothioate. As little as 1% of the O,O,O-trimethyl isomer protected rats from the intoxicating effects of the O,O,S-trimethyl isomer at doses as high as 200 mg/kg. Rat serum carboxylesterase and cholinesterase were inhibited for prolonged periods following a single oral dose of O,O,S-trimethyl phosphorothioate but the duration of inhibition was significantly less when the toxicant contained 1% of the O,O,O-trimethyl isomer.

2. Structure-Delayed Toxicity Relationships.

Introduction - Because of the unusual mode of action of O,O,S-trimethyl phosphorothioate, other structured analogs were examined for acute and delayed toxicity to rats. These compounds were also examined for anticholinesterase activities.

Delayed Toxicity of *O,O,S*-Trialkyl Phosphorothioates - Toxicological data presented in Table 10 for the different S-alkyl O,O-dimethyl and O,O-diethyl phosphorothioates reveals wide variability in the toxicity of these compounds to rats, with LD_{50} values ranging from 12 to >750 mg/kg. The most toxic compound was O,O-dimethyl S-propyl phosphorothioate (3), followed by either the O,O-dimethyl S-methyl (1) or S-ethyl analog (2). Change in the size of the S-alkyl moiety had a profound effect on rat toxicity and the S-hexyl analog (6) showed no toxicity at 750 mg/kg. Thus, increasing the number of carbon atoms of the S-propyl chain by three, i.e., from S-propyl to S-hexyl, reduced rat toxicity more than 62-fold.

TABLE 10. TOXICOLOGICAL PROPERTIES OF O,O-DIMETHYL AND O,O-DIETHYL S-ALKYLPHOSPHOROTHIOATES

$(RO)_2P(O)SR'$

			Toxicity to Rats			Cholinesterase Inhibition	
						ki (M^{-1} min^{-1})	
Compd	R	R'	Time frame for death, days after treatment	LD_{50} mg/kg	House fly LD_{50}, μg/g	BAChE	RSChE
1	CH_3	CH_3	4-22	15-20[a]	~95	0.43	5.16
2	CH_3	C_2H_5	3-11	18.2(7.3-26.7)[b]	103	0.44	3.34
3	CH_3	n-C_3H_7	1-3	12.0(7.7-13.9)	151	0.39	2.34
4	CH_3	n-C_4H_9	1-2	53.6(25.6-64.5)	162	0.51	2.04
5	CH_3	n-C_5H_{11}	1-3	311(283-335)	165	0.80	1.73
6	CH_3	n-C_6H_{13}	-	>750	261	0.44	2.76
7	CH_3	i-C_3H_7	1-7	108(101-115)	239	0.36	0.20
8	CH_3	t-C_4H_9	2-6	63.4(49.2-104)	301	17.8	28.7
9	C_2H_5	CH_3	1-7	47.1(42.6-50.3)	126	105	65.3
10	C_2H_5	C_2H_5	1-8	50-85[a]	-	-	-
11	C_2H_5	n-C_3H_7	1-3	48.5(37.5-71.6)	197.7	-	-
12	C_2H_5	i-C_3H_7	1	179(166-192)	223	144	99.8
13	C_2H_5	t-C_4H_9	1-3	127(112-230)	247	1490	14.4
14	C_2H_5	n-C_6H_{13}	-	>350	-	-	-

a/ Data from Mallipudi et al. (1979)

b/ Parenthetical values are 95% confidence limits.

The *S*-alkyl *O*,*O*-diethyl phosphorothioates were generally less toxic to rats than the *O*,*O*-dimethyl esters and in those cases where the *S*-alkyl moiety was the same the *O*,*O*-diethyl esters were 1.6 to 2.8-fold less toxic than the corresponding *O*,*O*-dimethyl ester. As in the case of the dimethyl esters, compounds with small *S*-alkyl groups showed the highest rat toxicities.

In general, rats treated with the compounds listed in Table 10 showed signs of delayed intoxication as previously described for *O*,*O*,*S*-trimethyl phosphorothioate. For example, typical symptoms of poisoning were weight loss, diarrhea, uncontrolled urination, and bleeding from the mouth and nose. Delayed toxicity is evident in the data presented in Table 11 for *O*,*O*-dimethyl *S*-isopropyl phosphorothioate (*7*) where death occurred over a period of 7 days. Extended weight loss was the most obvious sign of poisoning and even at the sublethal dose of 90 mg/kg substantial loss in weight was observed over 7 days. These animals eventually gained weight, however, and survived the treatment. Weight loss, diarrhea and bleeding were most apparent on the fourth and fifth days following treatment. At the high dosages of 130 and 140 mg/kg, most of the animals died within 24 hours after treatment. At these dosages it was difficult to distinguish between delayed and acute intoxication.

While delayed toxicity was observed in rats treated with virtually all of the compounds listed in Table 10, particularly at dosages near the LD_{50} value, in some cases death was rapid with rats showing typical cholinergic signs of poisoning. For example, out of 5 rats treated with 15 mg/kg *O*,*O*-dimethyl *S*-propyl phosphorothioate (*3*), 2 rats died within 24 hours and another on the second day. Symptoms of poisoning appeared to be cholinergic with animals showing hyperactivity, tremors, difficulty in breathing and eventually tetany. At 10 mg/kg, one rat died on the second day and two on the third day.

Cholinesterase Inhibition - Bimolecular rate constants (k_i) for the inhibition of bovine erythrocyte acetylcholinesterase (BAChE) and rat serum cholinesterase (RSChE) are given in Table 10. All of the trialkyl phosphorothioate esters were poor inhibitors of these two cholinesterases and, further, the *O*,*O*-dimethyl esters were essentially devoid of anticholinesterase activity. The absence of anticholinesterase activity for the dimethyl esters is particularly significant because of the high rat toxicity of some of these compounds.

The *O*,*O*-diethyl phosphorothioates were slightly stronger anticholinesterases than the dimethyl analogs with highest activity observed for *O*,*O*-diethyl *S*-*t*-butyl phosphorothioate (*12*). This compound, however, was one of the least toxic compounds in the series.

Summary - The *O*,*O*-dimethyl *S*-alkyl phosphorothioate esters containing small *S*-alkyl moieties (methyl, ethyl, *n*-propyl) were highly toxic to the rat. However, an increase in the *S*-alkyl chain length beyond propyl caused a precipitous drop in rat toxicity. Of the smaller *S*-alkyl *O*,*O*-dimethyl esters, the *S*-methyl and *S*-ethyl analogs clearly caused delayed toxic effects with death occurring at long time intervals after treatment. In contrast, rats

TABLE 11. TIME OF DEATH OF RATS TREATED ORALLY WITH DIFFERENT AMOUNTS OF *O,O*-DIMETHYL *S*-ISOPROPYL PHOSPHOROTHIOATE

Dose mg/kg	No. of rats treated	Time (days) of Occurrence of Death								Total rats killed	% Killed
		1	2	3	4	5	6	7	8-25		
0	4									--	0
90	5									--	0
100	7					1				1	14.28
110	7	1		1	1	1	1			5	71.42
120	5	2					1	1		4	80
130	5	3		1			1			5	100
140	5	3				1	1			5	100

treated with the S-n-propyl analog, the most toxic ester, died relatively quickly and it was difficult to differentiate acute from delayed effects.

It is apparent from anticholinesterase measurements that the dimethyl esters are inactive as cholinesterase inhibitors, being 10^5- to 10^6-fold less effective than such strong inhibitors as paraoxon and tetraethyl pyrophosphate. Further, the bimolecular rate constants (k_i) for the inhibition of either BAChE or RSChE were essentially the same for all of the straight chain O,O-dimethyl S-alkyl esters (CH_3 to n-C_6H_{13}). In contrast, the range in rat toxicity for these compounds was 12 to >750 mg/kg. The striking difference between rat toxicity and anticholinesterase activity suggest a non-cholinergic mechanism of intoxication for these compounds.

3. Delayed Toxicity and Delayed Neurotoxicity of Phosphoro- and Phosphonothioate Esters.

Introduction - Simple dialkyl phenylphosphonothioates O,O-diethyl phenylphosphonothioate, its corresponding oxon, and O,O-diethyl 4-chlorophenylphosphonothioate, impurities present in technical grade ethyl leptophos [O-(4-bromo-2,5-dichlorophenyl) O-ethyl phenylphosphonothioate], were found to cause delayed neurotoxicity in hens (see Section G-3). The mode of action of delayed neurotoxic compounds is still uncertain but clinical symptoms associated with delayed neurotoxicity are not seen until 8 to 14 days after exposure to the toxic agent. The delayed toxic response described in rats treated with the simple trialkyl phosphorothioates suggested the possibility that some of those compounds may cause delayed neurotoxicity in hens. It was also possible that dialkyl phenylphosphonates may cause delayed toxicity in rats. Therefore, trimethyl phosphate, trimethyl phosphorothioates and a series of methyl and ethyl esters of methyl-, ethyl- and phenylphosphonates and phosphonothioates were examined for delayed neurotoxicity to hens and delayed toxicity to rats. This section describes the results of this study.

Toxicological Results - A comparison of the acute and delayed toxicity to rats and the delayed neurotoxicity to hens of a series of simple O-alkyl and S-alkyl organophosphorus esters is shown in Table 12. All of the O,O-dialkyl phosphonothioates, phosphorothioates, and the corresponding oxons were relatively nontoxic to rats, i.e., LD_{50} values were greater than the 150 to 450 mg/kg tested. Each of the O,S-dialkyl phosphonothioate esters, however, was highly acutely toxic to rats. Two of these compounds, O,S-dimethyl methylphosphonothioate (XII) and O,S-diethyl ethylphosphonothioate (XV), with acute LD_{50} values of 3 and 8 mg/kg, respectively, were even more toxic than most organophosphorus insecticides. The rat LD_{50} for parathion is 4 to 13 mg/kg.

Acute toxicity was based on mortality 48 hours after treatment. When the holding period was extended to 25 days, two of the compounds O,O,S-trimethyl phosphorothioate (III) and O,S-diethyl ethylphosphonothioate (XV), showed delayed toxic activity (Table 13). The acute and delayed LD_{50} values for XV of 8 and 7 mg/kg, respectively, were statistically the same. Most of

TABLE 12. DELAYED NEUROTOXICITY IN THE HEN COMPARED WITH DELAYED TOXICITY IN THE RAT.

		Rat LD_{50}		Hen	
		single oral doses		10 daily i.p. doses	
No.	Compound	acute toxicity[a] (mg/kg)	delayed toxicity[b]	MED[c] (mg/kg)	delayed neuro-toxicity
I	$(CH_3O)_3P{=}S$	>300	-	50	-
II	$(CH_3O)_3P{=}O$	>300	-	50	-
III	$(CH_3O)_2P(O)SCH_3$	200[d]	15-20[d]	20	-
IV	$C_6H_5P(S)(OCH_3)_2$	>450	-	50	-
V	$C_6H_5P(O)(OCH_3)_2$	>450	-	50	-
VI	$C_6H_5P(O)(OCH_3)(SCH_3)$	55.3 (27.4, 72.9)	-	50	-
VII	$C_6H_5P(S)(OC_2H_5)_2$	>450	-	25	+
VIII	$C_6H_5P(O)(OC_2H_5)_2$	>450	-	25	+
IX	$C_6H_5P(O)(OC_2H_5)(SC_2H_5)$	75[g]	-	50	-
X	$CH_3P(S)(OCH_3)_2$	>300	-	50	-
XI	$CH_3P(O)(OCH_3)_2$	>150	-	50	-
XII	$CH_3P(O)(OCH_3)(SCH_3)$	3.3 (2.8, 4.0)	-	NE[e]	-
XIII	$C_2H_5P(S)(OC_2H_5)_2$	>450	-	50	-
XIV	$C_2H_5P(O)(OC_2H_5)_2$	>150	-	50	-
XV	$C_2H_5P(O)(OC_2H_5(SC_2H_5)$	7.8 (7.0, 8.6)	7.2 (6.5, 7.5)	20[f]	-

[a] Highest dose tested or LD_{50} with limits determined by probit analysis after 48 h; [b] LD_{50} determined after 25 days with limits from probit analysis; [c] Minimum effective dose for delayed neurotoxic compounds or highest dose tested for non-delayed neurotoxic compounds; [d] Data from Mallipudi et al., 1979; [e] Not examined; [f] Treated only 5 days because of high acute toxicity. [g] Limits not available.

the rats treated with XV lost weight for the first two or three days after treatment. Typical weight loss results are shown in Table 14. Rats that survived the first 48 hours and lost less than 25% of their initial body weight recovered completely. Their rate of weight gain by day 10 even exceeded that of control animals. However, rats that survived the first 48 hours but thereafter lost more than 25% of their initial body weight died within a few days. The weight loss varied from 34 to 37% at the time of death. Signs of intoxication for the first two days included diarrhea, excessive urination, and bleeding from the nose, mouth, and ears. All of the signs of intoxication except diarrhea had subsided within 48 hours in all of the treated rats.

When each of the compounds in Table 12 was examined for delayed neurotoxic activity in hens, only O,O-diethyl phenylphosphonothioate (VII) and its oxon (VIII) caused any ataxia up to 60 days after treatment. These two compounds had previously been found to cause severe ataxia in hens given single oral doses of 150 to 250 mg/kg (see Section G-3). Compounds III and XV, which were delayed toxic to rats, produced no symptoms of delayed neurotoxicity in hens treated with daily doses of 20 mg/kg for 10 and 5 days, respectively. Each of the O,S-dialkyl phosphonothioates, however, did produce acute cholinergic symptoms of intoxication within 10 minutes after treatment. Compound XV was so acutely toxic that three injections, each of 20 mg/kg, of atropine sulfate were administered at 15 minute intervals for survival.

Summary - A comparison of the toxic properties of those compounds that are delayed toxic to rats with those that are delayed neurotoxic to hens suggests that the two types of toxicity are evidently different. At least compounds that cause delayed toxicity in rats are not delayed neurotoxic to hens and vice versa. Of the fifteen compounds examined in the present study, only O,O-diethyl phenylphosphonothioate and its oxon, O,O-diethyl phenylphosphonate, were delayed neurotoxic.

O,S-Diethyl ethylphosphonothioate produced cholinergic symptoms in rats and most of the rats lost weight for the first two or three days. As the cholinergic symptoms subsided, rats that lost less than 25% of their initial body weight completely recovered while rats that lost more than 25% of their initial weight died. Death occurred up to 6 days after treatment when the weight loss was 34 to 37%. At this time, the data do not permit direct comparison between the delayed toxicity of O,O,S-trimethyl and triethyl phosphorothioate with that of O,S-diethyl ethylphosphonothioate. The symptomology of poisoning appears to indicate a possible similarity between the effects caused by the two types of compounds.

Each of the compounds examined in this study are potential or demonstrated impurities in a large number of phosphate or phosphonate pesticides. The safety of such pesticides warrants considerable investigation into the effects of these compounds on the toxicity of the parent pesticide for at least three reasons. First, O,O,S-trimethyl phosphorothioate has been shown to potentiate the acute toxicity of malathion to mammals (see Section E), and O,O-diethyl phenylphosphonothioate and its oxon potentiate the delayed

TABLE 13. DELAYED TOXICITY OF O,S-DIETHYL ETHYLPHOSPHONOTHIOATE ADMINISTERED ORALLY TO RATS

dose, mg/kg	treated	time (days) of occurrence of death							total rats killed	% killed
		1	2	3	4	5	6	7-25		
12.5	6	6							6	100
10	12	10							10	83
9	12	9		1	1				11	92
7.5	12	4	1						5	42
6	12	3	1				1		5	42
5	12	0							0	0

TABLE 14. PERCENT WEIGHT LOSS IN RATS TREATED WITH O,S-DIETHYL ETHYLPHOSPHONOTHIOATE

dose, mg/kg	rat no.	time (days) after treatment									
		1	2	3	4	5	6	7	8	9	10
9	1	died									
	2	died									
	3	died									
	4	died									
	5	13	27	34	died						
	6	died									
6	1	died									
	2	11	19	23	21	8	3				+17
	3	died									
	4	11	18	25	31	37	died				
	5	11	19	18	13	3	+8				+20
	6	died									
0	1	+3	+9	+15	+20	+26					+39
	2	+4	+13	+18	+24	+23					+44

neurotoxicity of ethyl leptophos to hens (see Section G-3). Second, *O,S*-dimethyl methylphosphonothioate and *O,S*-diethyl ethylphosphonothioate are more acutely toxic than most organophosphate pesticides in use. Third, the mode of action associated with the delayed toxic and delayed neurotoxic effects of *O,O,S*-trimethyl phosphorothioate, *O,S*-diethyl ethylphosphonothioate, *O,O*-diethyl phenylphosphonothioate, and *O,O*-diethyl phenylphosphate is not known and therapeutic treatment is not available.

4. Mode of Action of Delayed Toxic Action of *O,O,S*-Trimethyl Phosphorothioate.

Introduction - Efforts to determine the mode(s) of action of *O,O,S*-trimethyl phosphorothioate have focused on several areas. These include the effects of this compound on serum electrolyte, serum enzyme and urinary electrolyte levels; determinations of elimination, respiration and tissue distribution of radiochemical label in animals treated with ^{14}C-*O,O,S*-trimethyl phosphorothioate (labeled in the OCH_3 moiety); pathological studies of tissue damage following treatment with the compound; and the definition of pharmacokinetic parameters through the use of cannulae implanted in the exterior jugular vein of test animals. This section describes the results of these experiments.

Studies on the Pathological Effects of *O,O,S*-Trimethyl Phosphorothioate - Pathological studies carried out in conjunction with Dr. Herbert Braunstein, Director of San Bernardino County Medical Center, have indicated both primary and secondary morphological changes associated with *O,O,S*-trimethyl phosphorothioate intoxication.

Tissue samples were obtained at various time intervals following oral treatment from rats treated at doses of 30-200 mg/kg (corn oil carrier) with the compound. The findings for these animals may be summarized as follows:

Primary Changes - Villi of the small intestine show swelling in some cases with surface epithelial damage. Eventually, the villi shrink to small, club-like projections. At 200 mg/kg, areas of widely scattered and extensive liver necrosis are found. In some cases, this damage may be enough to account for death. In at least one case, the liver appeared to be in the process of regeneration at 48 hours.

Primary or Secondary Changes - Kidney samples show fatty deposits in and damage to the proximal tubuals, suggesting a nephrotoxic effect at the 80 mg/kg level and above. At high to moderate dosage, i.e., 80-200 mg/kg, adrenals show severe congestion. In some cases, this is almost hemorrhagic in nature. At all but the lowest dosages, the heart shows moderate congestion with some necrosis of the myocardium.

Secondary Changes - In a number of animals, we have observed severe to moderate pneumonia in the lungs and bronchia. In some cases, fatty deposits in the liver are found which may arise from malnutrition. Congestive changes in the liver, heart and lungs may relate to anoxia with resulting necrosis of the tissues.

The Effect of *O,O,S*-Trimethyl Phosphorothioate on Blood and Urine Parameters - Using animals cannulated in the exterior jugular vein for serial blood sampling, nonhemolyzed blood samples may be routinely obtained. Samples of blood taken after treatment with corn oil (carrier) or after treatment with 60 mg/kg *O,O,S*-trimethyl phosphorothioate (in corn oil) were analyzed for a variety of functions and parameters to determine what effect, if any, is brought about by intoxication. The results are summarized below and in Table 15.

Serum levels are stable for serum Na$^+$, Cl$^-$, Ca$^+$, CO$_2$ and albumin both before and after treatment with both carrier and carrier containing the *O,O,S*-trimethyl phosphorothioate. Serum K$^+$ levels show a gradual decrease following intoxication, but recover to normal levels in most cases for samples taken near the time of death. In separate studies, the urinary output of Na$^+$ and Cl$^-$ were found to dwindle to virtually zero following intoxication. Although the levels of urinary K$^+$ also drop at first following intoxication, these levels partially recover over time.

Examination of serum glutamic-oxalacetic transaminase levels in control animals show some variation between individual animals with relatively stable levels for a single animal. Following intoxication with 60 mg/kg *O,O,S*-trimethyl phosphorothioate, elevation of levels occurs in some but not all animals after 48 hours.

Serum glutamic-pyruvic transaminase levels in control animals were stable for individual cases showing little variation between animals. Following intoxication at 60 mg/kg, slight elevation was observed at 24 hours for several animals with levels returning to normal at or near death. In one case, an animal showed a large elevation (12 times normal) of the serum GPT level at 24 hours after treatment followed by a serum GOT level elevation (3 times normal) at 49 hours.

Blood samples from animals treated with 60 mg/kg *O,O,S*-trimethyl phosphorothioate ± 3.5% of the antagonist, *O,O,O*-trimethyl phosphorothioate were used for evaluation of hematological parameters. Samples from the intoxicated (nonantagonized) animals show severe hemato-concentration of the blood. Red blood cell counts were elevated with the hematocrit (% red cells/total blood volume) increased on average of 43.8% over those for the antagonized animals. In addition, the white blood cell count is depressed by approximately 53% for the nonantagonized animals. It seems likely that the cause of this concentration is directly related to fluid loss through diarrhea and excessive urination.

Metabolism of *O,O,S*-Trimethyl Phosphorothioate - The metabolism of [CH$_3$O-^{14}C]*O,O,S*-trimethyl phosphorothioate has been followed by tissue distribution, elimination and respiration or radiochemical label following oral treatment, as well as by pharmacokinetic procedures utilizing rats cannulated in the exterior jugular vein. Following treatment at 38 mg/kg, 63% of the total applied radioactivity was excreted in urine in 49 hours. Feces contained an additional 2% of the total radioactivity while respired

TABLE 15. SUMMARY OF RESULTS FOR LEVELS OF SERUM AND URINE FUNCTIONS FOLLOWING TREATMENTS WITH CARRIERS OR CARRIERS PLUS 60 mg/kg O,O,S-TRIMETHYL PHOSPHOROTHIOATE.

Functions measured	Results
Serum: Na^+, Cl^-, Ca^+, CO_2, albumin	Stable following treatment with carrier or carrier plus toxicant.
Urine: Na^+ and Cl^-	Levels (meq/l) drop drastically to virtually zero following intoxication.
Serum: K^+	Stable following carrier treatment. Drops by as much as 50% following treatment with toxicant. Regains normal levels at or near death.
Urine: K^+	Following intoxication, levels (meq/l) drop by as much as 60% (70-80 hours) but rise to levels approximately 35% below normal at 120 hours.

CO_2 accounted for 8.5% of the applied dose. See Figures 1 and 2 for relative excretion (in dpm/hour vs time following treatment) of ^{14}C-label via urine and CO_2, respectively. Feces account for the excretion of approximately 1% of the total applied radioactivity with 97% of this amount eliminated within 24.5 hours.

Approximately 2% of the applied radioactivity remains in the major organs of a treated animal at 48-50 hours with highest levels in the liver, contents of the large intestine, adrenals, kidneys, spleen and tissue of the small intestine, in decreasing order.

Pharmacokinetic Studies - Rats were cannulated in the exterior jugular vein approximately one week prior to treatment. Blood samples were collected following intraperitoneal, intravenous or oral treatment with $[CH_3O-^{14}C]$O,O,S-trimethyl phosphorothioate with or without the antagonist, O,O,O-trimethyl phosphorothioate. For i.p. administration, maximum levels were obtained at 4-6 minutes after treatment, while oral levels require 10-30 minutes to reach maximum. Blood levels of radioactivity were similar for oral and i.p. treatment at 24 hours but lower for i.v. treatment. Blood levels of total radioactivity are similar for treatment with or without the antagonist. Figure 3 shows representative curves for i.v., i.p. and oral treatments at 60 mg/kg.

Summary - Animals intoxicated with O,O,S-trimethyl phosphorothioate show several physiological and pathological changes. Most important of these are: lowered serum K^+ levels which may lead to myocardial damage; severe hemato-concentration resulting from diarrhea and excessive urination leading to stress on the circulatory system; damage to the liver and kidneys at moderate to high doses of the material.

Initial ^{14}C-labeling studies show a rapid uptake and stabilization in the body of radioactivity with slow decline of total radioactivity up to and including 3 hours following treatment. As expected, relative levels are much higher following i.p. and i.v. treatments when compared to oral treatment. However, at 24 hours, a higher percentage of the initial maximum ^{14}C-level in the blood remains following oral treatment when compared to levels from i.p. treatment.

Metabolism leads primarily to excretion in the urine with a half-life of 20-24 hours. Of the 63% of total applied radioactivity excreted in the urine, only 1.6% is isolated as unchanged material. Little of the ^{14}C-label is excreted as CO_2 or in fecal matter. Identification of the metabolites is proceeding.

Figure 1. EXCRETION OF ^{14}C-LABEL IN RAT URINE AFTER $(C^{*}H_3O)_2P(O)SCH_3$ TREATMENT

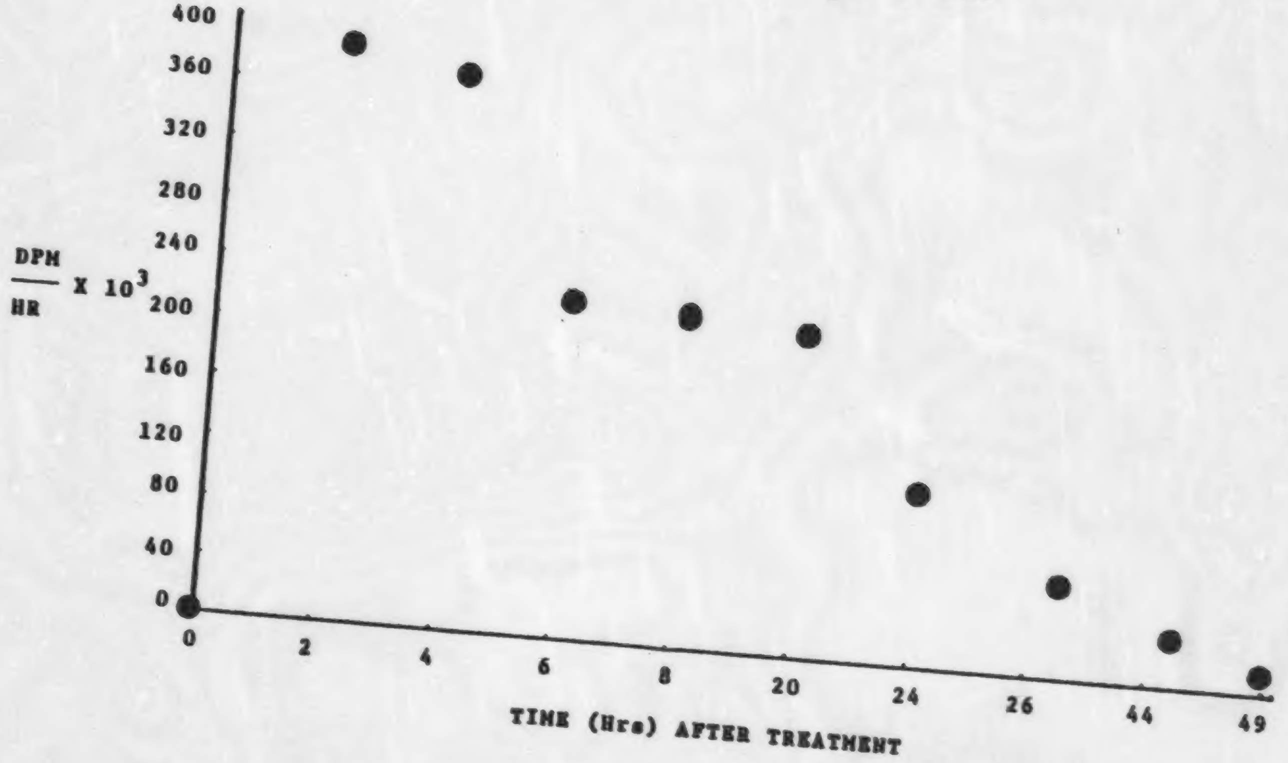
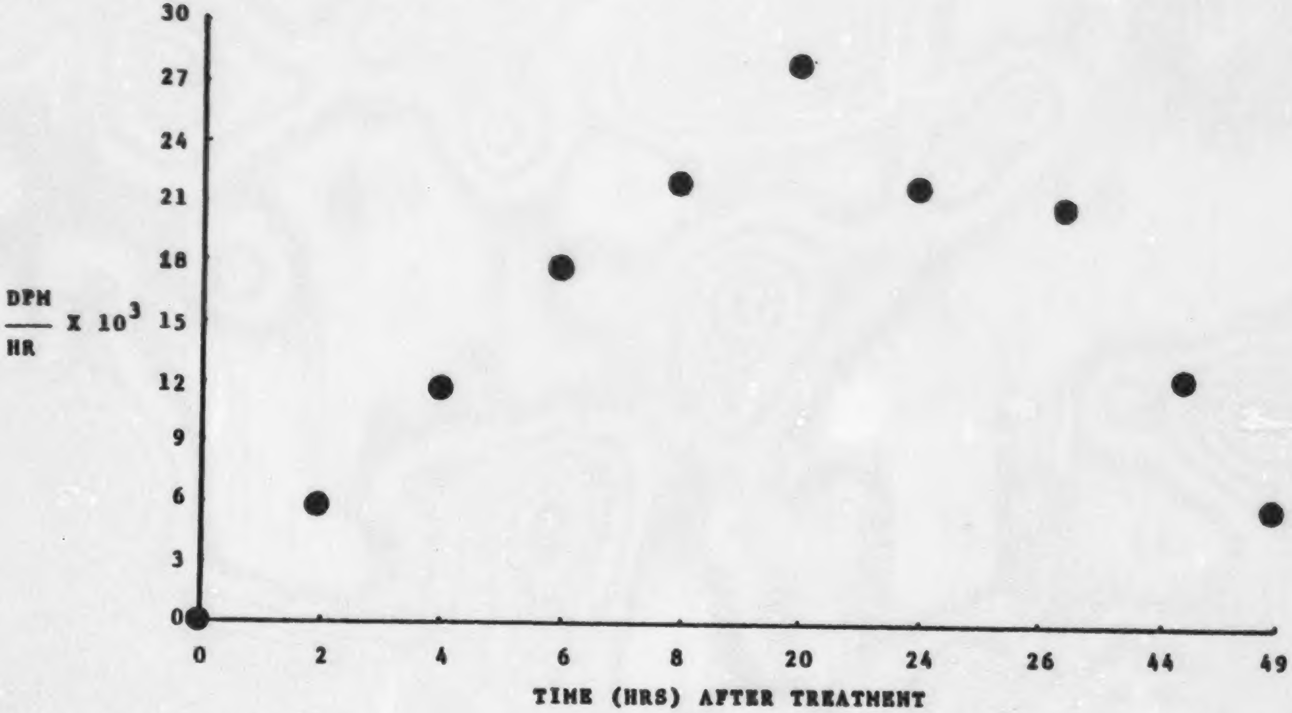


Figure 2. RESPIRATION OF ^{14}C -LABEL IN RAT AFTER $(C^{*}H_3O)_2P(O)SCH_3$ TREATMENT



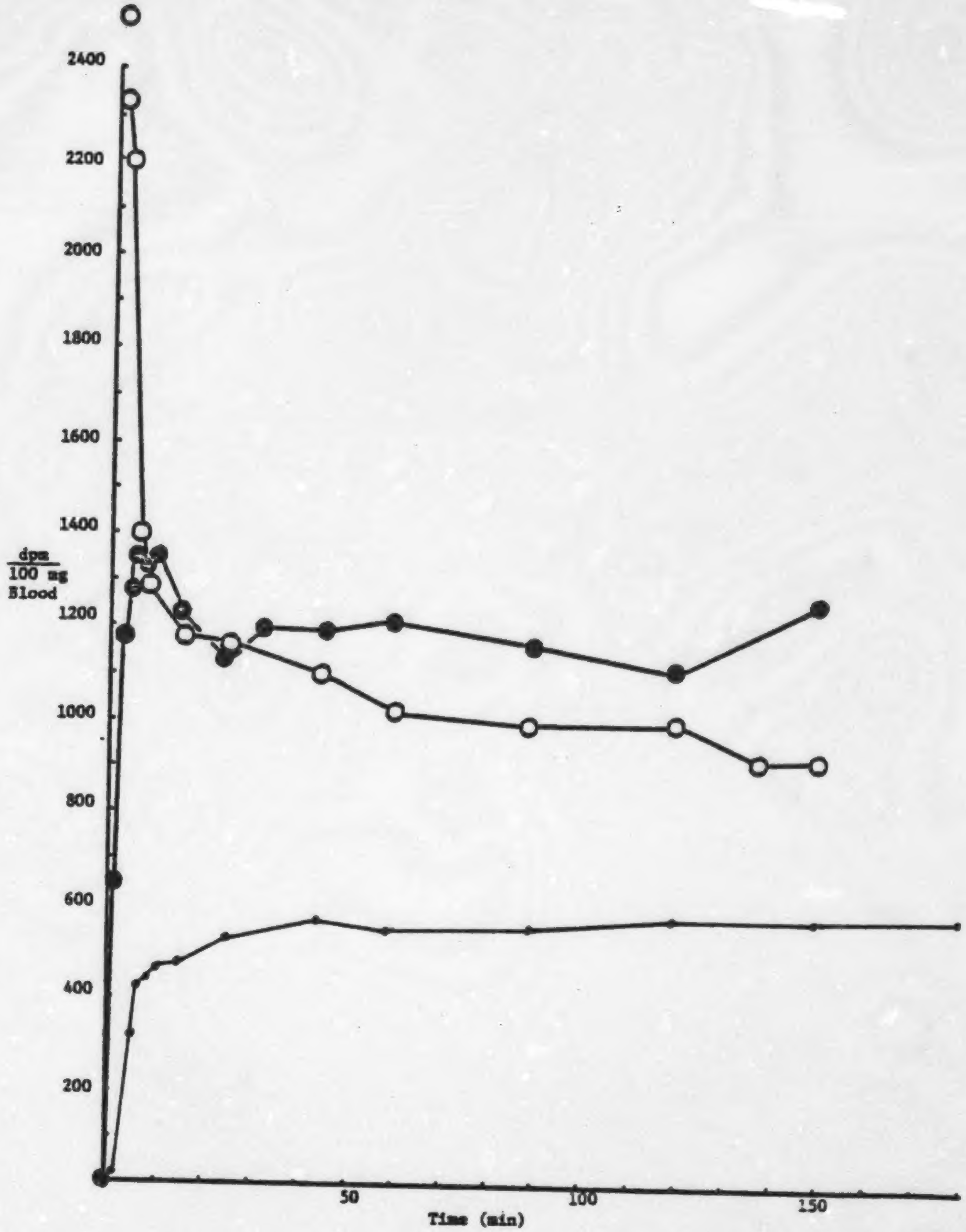


Figure 3. Blood radioactivity levels (dpm/mg) vs time following treatment with ^{14}C-O,O,S-trimethyl phosphorothioate plus 6-7% (w/w) O,O,O-trimethyl phosphorothioate. (○) i.v. - 60 mg/kg; 9.375 x 10^6 dpm/kg (propylene glycol carrier); (●) i.p. - as above; (•) oral - as above.

G. DELAYED NEUROTOXICITY OF ORGANOPHOSPHORUS ESTERS

1. Effect of Structure on the Delayed Neurotoxicity of *O*-Alkyl *O*-Aryl Phenylphosphonothioate Analogs Related to Leptophos.

Introduction - The discovery of the potential of some organophosphorus compounds to cause an insidious neuropathic anomaly, termed delayed neurotoxicity, has necessitated careful screening of potential organophosphorus pesticides for such delayed neurotoxic activity[1]. An earlier incidence of delayed neurotoxicity involved the use of leptophos [PHOSVEL or *O*-(4-bromo-2,5-dichlorophenyl) *O*-methyl phenylphosphonothioate] for control of cotton pests in Egypt[2]. Others[3,4,5] have conclusively demonstrated that leptophos, leptophos oxon, and desbromoleptophos are capable of producing delayed neurotoxicity in hens. A systematic study of pesticidal activity versus delayed neurotoxic potential of structural analogs of leptophos was therefore conducted.

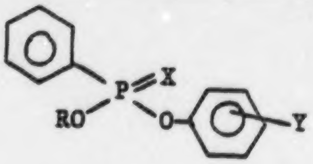
Toxicological Properties - Data for the acute toxicity of the various leptophos analogs to house flies and white mice, and delayed neurotoxicity to hens are given in Table 1. Several compounds were highly toxic to house flies; the most toxic were EPN (14) and its methoxy analog (15). These two compounds also were acutely highly toxic to mice and hens, and both produced severe ataxia in hens when their acute toxicity was counteracted by three intramuscular injections of atropine.

All of the monochloro- and dichlorophenyl *O*-methyl phenylphosphonothioate analogs (1-9) caused ataxia in hens. Compared to leptophos the dichlorophenyl esters were more delayed neurotoxic while the monochlorophenyl esters (5-7) were less delayed neurotoxic. The unsubstituted phenyl ester (10) was not delayed neurotoxic at a single oral dose of 500 mg/kg. The 2,5-dichlorophenyl ester (3 or desbromoleptophos), with a single minimum effective dose of 23-26 mg/kg, was the most delayed neurotoxic analog examined. This compound has been reported to be one of the major photolytic conversion products of leptophos[5].

Substitution of the *O*-methyl group of leptophos by *O*-ethyl gave an analog (19) which did not show delayed neurotoxic activity, i.e., symptoms of delayed neurotoxicity were not observed at a single oral dose as high as 1000 mg/kg. Similarly, the *O*-ethyl analog (11) of desbromoleptophos also was not delayed neurotoxic at 1000 mg/kg. In this case, the reduction in activity was substantially more significant owing to the greater delayed neurotoxicity of desbromoleptophos compared to leptophos. The *O*-propyl (12) and *O*-*n*-butyl (13) analogs of desbromoleptophos were not delayed neurotoxic at single oral doses of 500 and 333 mg/kg, respectively, the highest doses tested.

Examination of house fly toxicity data show that the ethyl analogs of leptophos (19) and desbromoleptophos (11) were as effective as leptophos against house flies. Further, 19 and 11, with mouse oral LD_{50} values of 75 and 153 mg/kg, were both less toxic to mice than leptophos.

TABLE 1. ACUTE AND DELAYED NEUROTOXICITY OF LEPTOPHOS ANALOGS TO HOUSE FLIES, MICE AND HEN CHICKENS



				Acute Toxicity		Neurotoxicity
Compound	X	R	Y	House Fly LD_{50} (μg/g)	Mouse (oral) LD_{50} (mg/kg)	Hen MED[a] (mg/kg)
Leptophos	S	CH_3	2,5-di-Cl-4-Br	13	65	+ 250-275
1	S	CH_3	2,6-di-Cl	430	105	+ 40-50
2	S	CH_3	2,5-di-Cl	10	95	+ 23-26
3	S	CH_3	2,4-di-Cl	30	240	+ 160-175
4	S	CH_3	2,3-di-Cl	34	>500	+ 100-175
5	S	CH_3	2-Cl	270	165	+ 300-325
6	S	CH_3	3-Cl	100	>500	+ 275-300
7	S	CH_3	4-Cl	>500	>500	+ 385-480
8	S	CH_3	3,4-di-Cl	32	100	+ 100-175
9	S	CH_3	3,5-di-Cl	5	200	+ 175-225
10	S	CH_3	H	>500	>500	- 500
11	S	C_2H_5	2,5-di-Cl	12	153	- 1000[b]
12	S	C_3H_7	2,5-di-Cl	>500	>500	- 500
13	S	C_4H_9	2,5-di-Cl	>500	>500	- 333
14	S	C_2H_5	4-NO_2	1	16	+ 100-125[b]
15	S	CH_3	4-NO_2	2	12	+ 100-125[b]
16	O	bis(dimethoxy)	—	>500	>500	- 500
17	O	bis(2,5-di-Cl-phenyl)	—	>500	>500	- 500
18	S	bis(2,5-di-Cl-4-Br-phenyl	—	>500	>500	- 500
19	S	C_2H_5	2,5-di-Cl-4-Br	9	75	- 1000[b]

[a] Minimum effective dose producing ataxia

[b] Atropinized

Compound 18 is a reported impurity of technical leptophos and compounds 16 and 17 are potential photolytic oxidation products of reported impurities. None of these compounds caused acute or delayed toxic symptoms in any of the test animals at 500 mg/kg.

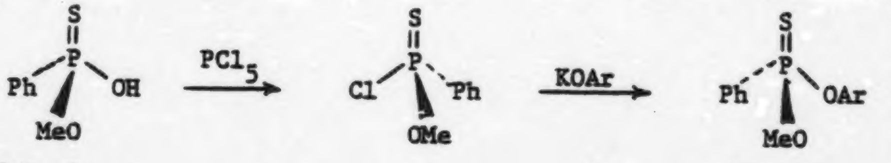
Summary - Analogs of leptophos [O-(4-bromo-2,5-dichlorophenyl) O-methyl phenylphosphonothioate] were examined for acute toxicity to mice and house flies, and for delayed neurotoxic activity in adult hens following administration of a single oral dose. Development of ataxia after recovery from acute poisoning was the criterion for delayed neurotoxic activity. All mono- and dichlorophenyl analogs were delayed neurotoxic, and 2,5-dichlorophenyl analog being the most potent delayed neurotoxic compound tested. Substitution for the methyl by an ethyl group abolished delayed neurotoxic activity in both leptophos and the 2,5-dichlorophenyl analog at doses of 1000 mg/kg. The n-propyl and n-butyl analogs of the latter were also nondelayed neurotoxic at 500 mg/kg and 333 mg/kg, respectively. Substitution of methyl for ethyl in EPN [O-p-nitrophenyl O-ethyl phenylphosphonothioate] did not alter its delayed neurotoxicity.

2. Effect of Chirality on the Toxicological Properties of Leptophos and Desbromoleptophos.

Introduction - It is well known that chirality at the phosphorus atom of an organophosphorus ester often has a significant effect on the biological activity of the ester. The effect of phosphorus chirality on the delayed neurotoxicity of the enantiomers of EPN (O-ethyl O-p-nitrophenyl phenylphosphonothioate) and the corresponding oxons has been described[6]. The dextrorotatory isomer was several-fold more acutely toxic to hens, house flies, and the rice stem borer while the levorotatory isomer was more delayed neurotoxic to hens. Such differences in the toxicological properties of the chiral isomers suggest that the active site associated with delayed neurotoxicity is stereochemically different from the site associated with acute toxicity, i.e., acetylcholinesterase.

This section is concerned with the resolution and determination of the absolute configuration of the chiral isomers of leptophos and desbromoleptophos, and the toxicological properties of these compounds.

Determination of Absolute Configuration - The synthesis of the chiral isomers of leptophos and desbromoleptophos was carried out according to the following reactions for one of the isomers. The procedure is essentially



(1)

$(\underline{S})_P(-)$, 1a

$(\underline{R})_P(+)$

3b, Ar = 4-Br-2,5-di-Cl-phenyl
4b, Ar = 2,5-di-Cl-phenyl

the same as described earlier for the synthesis of the chiral isomers of fonofos (see Part A).

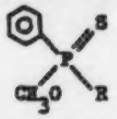
The absolute configuration of (-)-*O*-methyl phenylphosphonothioic acid (*1a*) was established to be (*S*)$_p$ by x-ray diffraction analysis of the (-)-α-methylbenzylammonium salt of the acid (*2a*).

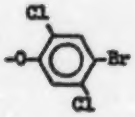
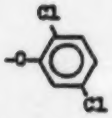
Owing to a pseudosymmetry problem, the determination of the absolute configuration of (-)-leptophos (*3a*) was not successful. However, assignment of configuration of the leptophos and desbromoleptophos enantiomers may be made on the basis of the known stereochemistry of reactions related to those described in equation 1. Previous work in this laboratory[7] showed that conversion of resolved *O*-ethyl ethylphosphonothioic acid to *O*-ethyl ethylphosphonochloridothioate by the action of phosphorus pentachloride occurred with inversion of the phosphorus center. Reaction of the chloridothioate with sodium benzenethiolate to give fonofos (*O*-ethyl *S*-phenyl ethylphosphonodithioate) also occurred with inversion. Assuming that inversion takes place in each step of the two-step reaction from *1a* to either *3b* or *4b*, the absolute configuration of these products is (*R*)$_p$, as indicated in equation 1. Conversely, *3a* and *4a* are (*S*)$_p$ (see Table 2).

Assessment of Optical Purity - The NMR spectrum of racemic *O*,*S*-dimethyl phenylphosphonothioate showed doublets for OCH_3 and SCH_3 protons at δ 3.8 and 2.1 ($J = 14$ Hz), respectively. Addition of the pseudo-contact shift reagent tris-[3-(trifluoromethylhydroxymethylene)-*d*-camphorato] europium (III) to the racemic material resulted in two doublets (separated by 2.4 Hz) for the SCH_3 protons. Treatment of the sodium salt of (-)-*O*-methyl phenylphosphonothioic acid (*1a*, α_D^{24} -21.0°) with dimethyl sulfate resulted in (-)-*O*,*S*-dimethyl phenylphosphonothioate (*5a*). Addition of the shift reagent to this material resulted in no separation of the SCH_3 doublet, indicating that the minus rotating ester was optically pure. Since the configuration of the phosphorus atom was not affected in the conversion of *1a* to *5a*, the starting acid *1a* also was optically pure.

Addition of tris [3-(trifluoromethylhydroxymethylene)-*d*-camphorato] europium (III) to leptophos oxon resulted in a 24-Hz splitting of the OCH_3 signal into two doublets. A sample of (*S*)p-leptophos (*3a*, $[\alpha]_D^{24}$ -46.26) was converted to the corresponding (-)-leptophos oxon (*6a*) by reaction with *m*-chloroperoxybenzoic acid. Addition of the shift reagent to *6a* resulted in two OCH_3 doublets centered at δ 3.8, separated by 24 Hz. These corresponded to 98% 6a and 2% 6b [(+)-leptophos oxon], indicating that 3a was at least 98% optically pure. Similarly, a sample of (*S*)p-desbromoleptophos (*4a*, $[\alpha]_D^{24}$ -23.03) was converted to its corresponding oxon (7a). Addition of the shift reagent to *7a* resulted in two OCH_3 doublets centered at δ 4.2, separated by 2.4 Hz. These corresponded to 72% *7a* and 28% *7b* [(+)-desbromoleptophos oxon], indicating that *4a* was only 72% optically pure. The conversion of fonofos to its oxon has been shown to occur with retention of configuration[7].

TABLE 2. PHYSICAL CONSTANTS AND VALUES OF SPECIFIC ROTATION IN DEGREES ARC FOR THE COMPOUNDS OF GENERAL STRUCTURE



	R	Abs Config.	mp or bp (mm), °C	n^{23}_D	Specific $[\alpha]^{24}_D$
1	-OH	Racemic	100 (0.02)	1.5772	-
1a		$(S)_P$	-	-	-21.0 (neat)
1b		$(R)_P$	-	-	+21.72 (neat)
2	$-O^{-}H_3\overset{+}{N}CH(CH_3)C_6H_5$	Racemic	142-144	-	-
2a		$(S)_P$	142-144	-	-17.68 (c 14.5)[a]
2b		$(R)_P$	142-144	-	+17.83 (c 10.75)[a]
3		Racemic	71-72	-	-
3a		$(S)_P$	88-90	-	-46.26 (c 10.0)[b]
3b		$(R)_P$	88-90	-	+46.13 (c 10.6)[b]
4		Racemic	46-47	-	-
4a		$(S)_P$	46-47	-	-23.03 (c 2.08)[b]
4b		$(R)_P$	46-47	-	+22.72 (c 2.24)[b]

[a] Specific rotation was measured in methanol.

[b] Specific rotation was measured in benzene.

Toxicological Properties - The (R)p(+) isomers of leptophos and desbromoleptophos were more toxic to house flies and mice than were the (S)p(-) isomers, although differences in toxicities were not large (Table 3). The largest difference in toxicity was observed for leptophos against house flies, where the (R)p(+) enantiomer was about 4-fold more active than the (S)p(-) enantiomer. In all other cases, the difference was substantially less than 2-fold; however, greater differences would be expected from optically pure desbromoleptophos. Overall, the acute toxicological data were similar to those reported for the (+) and (-) isomers of EPN[6] (ethyl O-p-nitrophenyl phenylphosphonothioate) and for the (R)p(+) and (S)p(-) isomers of fonofos[8] (see Part A). In all these cases, the (+)-rotating isomer showed higher activity.

Sufficient quantities of resolved leptophos were not available for a complete evaluation of its delayed neurotoxic activity. In a preliminary study the (S)p(-) enantiomer (3a) produced severe paralysis in hens given 100 mg/kg while the (R)p(+) enantiomer (3b) produced no effects (Table 4).

In a more extensive study with desbromoleptophos, little difference in delayed neurotoxic activity was observed between the (S)p(-) (4a) and the (R)p(+) (4b) enantiomers, as shown in Table 5. The severity of the ataxia, however, was much more pronounced in hens given the (S)p(-) enantiomer. This relatively slight difference in activity was attributed to the lack of complete optical purity of the desbromoleptophos enantiomers.

It is apparent that both the (S)p(-) isomers of leptophos and desbromoleptophos and the (-) isomers of EPN and EPN oxon are more delayed neurotoxic while the (R)p(+) isomers of leptophos and desbromoleptophos and the (+) isomers of EPN and EPN oxon are more acutely toxic. These findings add further support to the hypothesis that the biochemical lesion responsible for delayed neurotoxicity is separate from the lesion responsible for acute toxicity. Furthermore, the active site associated with organophosphorus induced delayed neurotoxicity must be stereochemically different from the acetylcholinesterase site associated with acute toxicity.

Summary - The chiral isomers of O-methyl phenylphosphonothioic acid, O-(4-bromo-2,5-dichlorophenyl) O-methyl phenylphosphonothioate (leptophos), and O-(2,5-dichlorophenyl) O-methyl phenylphosphonothioate (desbromoleptophos) were prepared and their absolute configurations established by x-ray diffraction analysis of the (-)-α-methylbenzylammonium salt. The absolute configurations of the enantiomers of leptophos and desbromoleptophos were assigned by relating them to the configurations of the corresponding O-methyl phenylphosphonothioic acids. The (R)p(+) isomers were more acutely toxic to the house fly and white mouse while the (S)p(-) isomers were more delayed neurotoxic to the hen.

3. Effect of Impurities on the Delayed Neurotoxicity of Ethyl Leptophos.

Introduction - In a preceding section on the delayed neurotoxicity of structural analogs related to leptophos, it was reported that substitution of the methoxy moiety with a longer chain alkoxy group, e.g., ethoxy or propoxy abolished delayed neurotoxic activity in hens at the highest doses

TABLE 3. ACUTE TOXICITY OF RACEMIC, $(\underline{R})_P(+)$, and $(\underline{S})_P(-)$ ISOMERS OF LEPTOPHOS AND DESBROMOLEPTOPHOS TO HOUSE FLIES AND MICE

Compound	LD_{50}	
	House fly (topical) µg/g	Mouse (oral) mg/kg
Desbromoleptophos		
(±)	11.4^a	54.9^a
$(\underline{R})_P(+)$	10.4^a	51.3^a
$(\underline{S})_P(-)$	17.3^b	63.7^a
Leptophos		
(±)	14.1^a	71.9^a
$(\underline{R})_P(+)$	5.7^b	64.4^a
$(\underline{S})_P(-)$	22.2^c	75.0^a

Values with common superscript are not significantly different, $P = 0.05$.

TABLE 4. THE DELAYED NEUROTOXIC EFFECT OF RACEMIC (±), (R)P(+), AND (S)P(-) LEPTOPHOS IN ATROPINIZED HENS

	(±)			(R)$_P$(+)			(S)$_P$(-)		
Dose mg/kg	hens dosed No.	death No.	delayed paralysis No.	hens dosed No.	death No.	delayed paralysis No.	hens dosed No.	death No.	delayed paralysis No.
50	5	0	0	-	-	-	-	-	-
100	5	0	3	2	0	0	2	0	2
150	5	0	5	-	-	-	-	-	-

TABLE 5. THE DELAYED NEUROTOXIC EFFECT OF RACEMIC, (R)$_P$(+), and (S)$_P$(-) DESBROMOLEPTOPHOS IN ATROPINIZED HENS

	(±)			R$_p$(+)			S$_p$(-)		
Dose (mg/kg)	No. Treated	Acute Toxicity	Delayed Neuro-toxicity	No. Treated	Acute Toxicity	Delayed Neuro-toxicity	No. Treated	Acute Toxicity	Delayed Neuro-toxicity
1	3	0	0	5	0	0	5	0	0
5	3	0	0	5	1	0	5	0	1
10	8	0	1	8	0	1	8	0	2
20	5	0	2	3	0	3	3	0	2
30	3	0	2	3	0	3	3	0	3
40	3	0	3	3	1	2	3	0	3
50	3	0	3	3	0	3	3	0	3

tested (see Section 1). The minimum effective dose (MED) for desbromoleptophos, the most potent delayed neurotoxic compound tested, was 25 mg/kg, yet neither ethyl leptophos [O-(4-bromo-2,5-dichlorophenyl) O-ethyl phenylphosphonothioate] nor desbromo ethyl leptophos [O-(2,5-dichlorophenyl) O-ethyl phenylphosphonothioate] showed delayed neurotoxic activity at 1000 mg/kg. Preliminary studies indicated a difference in the delayed neurotoxic activity between technical ethyl leptophos and the recrystallized material. This observation suggested a need for a more critical evaluation of the toxicological properties of ethyl leptophos. This section describes investigations pertaining to: (a) the delayed neurotoxicity of ethyl leptophos; (b) the effect of organophosphorus (OP) impurities in the technical grade material on delayed neurotoxicity; (c) the relationship of dose and time on delayed neurotoxicity of technical vs purified material with multiple doses; and (d) the inherent delayed neurotoxicity of significant OP impurities.

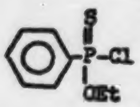
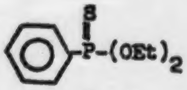
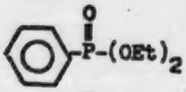
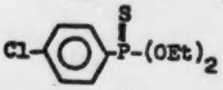
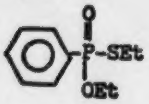
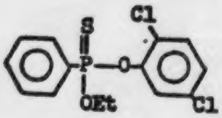
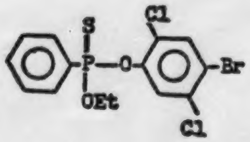
Composition of Ethyl Leptophos - The different compounds which were identified in technical ethyl leptophos are shown in Table 6. The principal method for structure elucidation of these compounds was by GC-MS. Structures of compounds for which synthetic standards were available gave identical CIMS fragmentation patterns to identified impurities and were further confirmed by GC retention times and TLC co-chromatography.

Table 6 shows the percent composition of technical ethyl leptophos before and after storage. The technical material was nearly 90% pure and was stable under the storage conditions of this experiment. Furthermore, all of the impurities measured were also stable except for O-ethyl phenylphosphonochloridothioate (peak 4).

Delayed Neurotoxicity of Technical vs Purified Ethyl Leptophos - A comparison of the delayed neurotoxic potential of technical vs purified ethyl leptophos as single oral doses to hens is shown in Table 7. The MED was 1000-1250 mg/kg for purified ethyl leptophos while the MED for the technical material was at most 750 mg/kg. The onset of ataxia in hens treated with purified material was also 5 to 10 days later than for hens given the technical compound. A statistical evaluation of these data, and that shown in Tables 8 and 9, was not conducted because some stages of ataxia were not always observed in all of the hens under similar treatment. The variation in response time among hens that did develop the same degree of ataxia was generally 2 to 5 days.

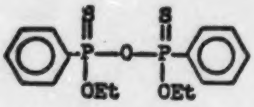
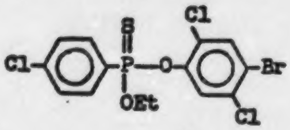
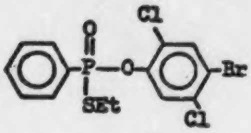
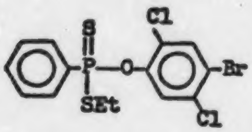
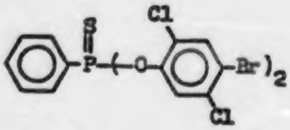
Table 8 shows a comparison of the same two materials as in Table 7 except that the hens were given daily oral doses of 10 or 50 mg/kg/day for periods of 24 or 45 days. Hens given 50 mg/kg/day of either material developed severe ataxia, stage T_4, during both treatment periods whereas none of the hens given 10 mg/kg/day showed any sign of ataxia. At those dose levels there appears to be no difference in the delay period between technical and purified materials. Surprisingly, however, hens given either material for 24 days developed ataxia 7 to 15 days earlier than hens under similar treatment for 45 days.

TABLE 6. CHEMICAL COMPOSITION OF TECHNICAL ETHYL LEPTOPHOS BEFORE AND AFTER STORAGE AT 40°C FOR 4.5 MONTHS AS DETERMINED BY GAS CHROMATOGRAPHY/MASS SPECTROSCOPY

Peak No.[a]	Structure	Percent Composition Unstored	 Stored
1	Unknown (non-phosphorus)	-	-
2	Unknown (non-phosphorus)	-	-
3	Unknown (non-phosphorus)	-	-
4		1.4	<0.1
5		1.5	1.7
6		0.1	0.1
7		<0.1	<0.1
8		<0.1	<0.1
10		<0.1	<0.1
12		89.5	89.5

continued next page

TABLE 6. (cont'd)

12b		<0.1	<0.1
12c		2.1	2.1
13b		0.6	0.8
13c		-	-
14	Unknown (Phosphorus)	-	-
15	Unknown (Phosphorus)	-	-
16		1.6	1.6
		Total 96.8-97.2	95.8-96.3

[a] Glc peaks

TABLE 7. DELAYED NEUROTOXIC ACTIVITY OF TECHNICAL VS PURIFIED ETHYL LEPTOPHOS AS SINGLE ORAL DOSES TO HENS

Compound	Dose mg/kg	Mean days after treatment when ataxia was observed			
		T_1[a]	T_2	T_3	T_4
Technical (89% pure)	1500	14	16	19	21
	1250	14	16	18	20
	1000	14	20	30	-[b]
	750	?	23	26	30
Purified (>99% pure)	1500	24	25	27	29
	1250	21	23	24	27
	1000	25	-	-	-
	750	-	-	-	-

---

[a] T_1 = clumsy; T_2 = drunken; T_3 = sitting on hocks; T_4 = unable to stand.

[b] This stage of ataxia was not observed.

TABLE 8. DELAYED NEUROTOXICITY OF TECHNICAL VS PURIFIED ETHYL LEPTOPHOS ADMINISTERED AS DAILY ORAL DOSES TO HENS

Compound	Dose (mg/kg)	No. days treated	Mean days after treatment when ataxia was observed			
			T_1[a]	T_2	T_3	T_4
Technical (89% pure)	10	24	-[b]	-	-	-
	50	24	-	37	40	52
	10	45	-	-	-	-
	50	45	-	49	63	67
Purified (>99% pure)	10	24	-	-	-	-
	50	24	-	36	38	49
	10	45	-	-	-	-
	50	45	-	43	52	64

---

[a] T_1 = clumsy; T_2 = drunken; T_3 = sitting on hocks; T_4 = unable to stand.

[b] This stage of ataxia was not observed.

TABLE 9. EFFECTS OF STORAGE AT 40°C ON THE DELAYED NEUROTOXICITY OF TECHNICAL ETHYL LEPTOPHOS ADMINISTERED ORALLY TO HENS

Compound	Dose (mg/kg)	No. treated	Mean days after treatment when ataxia was observed			
			T_1[a]	T_2	T_3	T_4
Technical	1250	3	14	16	18	20
	1000	3	14	21	29	31
	750	3	-[b]	23	25	30
Technical (stored)	1250	3	23	26	-	-
	1000	3	-	-	-	-
	750	3	-	-	-	-

---

[a] T_1 = clumsy; T_2 = drunken; T_3 = sitting on hocks; T_4 = unable to stand.

[b] This stage of ataxia was not observed.

Earlier, we reported that ethyl leptophos was not delayed neurotoxic to hens at 1000 mg/kg. The first stage of ataxia is often difficult to positively identify either clinically or histopathologically[9,10,11]. In this study, hens given 1000 mg/kg of purified ethyl leptophos, although slightly clumsy, were difficult to distinguish from control animals while hens given doses greater than 1000 mg/kg developed severe ataxia. It seems apparent that the highest dose examined in our previous study was just at threshold and positive ataxia was therefore not observed. It is also evident that some component of the technical material is enhancing its neurotoxicity.

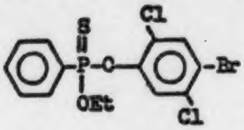
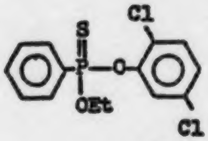
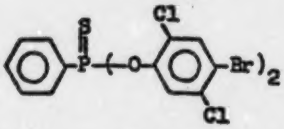
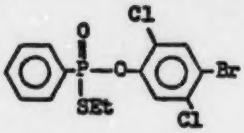
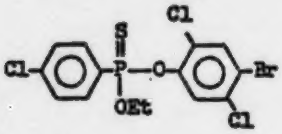
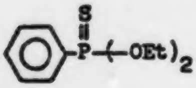
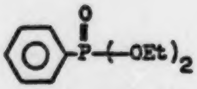
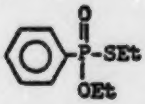
The difference in neurotoxic potential between technical and purified ethyl leptophos prompted an analysis of the chemical composition of the technical material and its stability during storage at 40°C for 4.5 months. As shown in Table 9, the MED for technical material after storage was 1250 mg/kg compared to at most 750 mg/kg for nonstored material. The delay period was also approximately 10 days longer for the stored material than for the nonstored material. When these values are compared with those for technical and purified ethyl leptophos shown in Table 7, the MED and delay period for technical material after storage is similar to purified ethyl leptophos. The reason for the change in delayed neurotoxic pattern is not readily evident in light of the apparent stability of the technical material during storage.

Delayed Neurotoxicity of Impurities - An analysis of the delayed neurotoxicity of organophosphorus impurities present in technical ethyl leptophos in excess of 0.1 percent is shown in Table 10. While none of the aryl esters tested except ethyl leptophos caused any sign of ataxia at the doses examined, each of the O,O-diethyl esters caused severe ataxia. The O,S-diethyl phenylphosphonothioate, however, was not neurotoxic at 500 mg/kg.

A more extensive study of the diethyl esters is summarized in Table 11. The MED for O,O-diethyl phenylphosphonothioate was 150 mg/kg and 100 mg/kg for its oxon analog, O,O-diethyl phenylphosphonate. O,O-Diethyl 4-chlorophenylphosphonothioate was neurotoxic at 150 mg/kg. The delay period for each compound was generally 10 to 12 days for the first signs of ataxia but complete paralysis developed within 2 to 6 days later. This delay pattern was similar to that observed for hens given technical ethyl leptophos, shown in Table 7. It now seems evident that the difference in delayed neurotoxicity between technical and purified ethyl leptophos is due to these diethyl phenylphosphonate impurities.

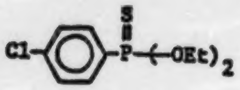
It is possible that the mixture of impurities present in technical ethyl leptophos is potentiating its delayed neurotoxicity but additional studies are necessary to establish the mechanism involved. Since these diethyl phenylphosphonate impurities are probably present in other O-ethyl phenylphosphonothioate pesticides, e.g. EPN, cyanofenphos, their effect on the delayed neurotoxic potential of such compounds should be investigated. The unexpectedly high neurotoxic activity observed for O,O-diethyl

TABLE 10. DELAYED NEUROTOXICITY OF SOME IMPURITIES IN TECHNICAL ETHYL LEPTOPHOS ADMINISTERED ORALLY TO HENS

Compound	No. Hens treated	Dose (mg/kg)[a]	No. ataxic[b]
	3	1250	3
	3	1000	0
	3	1000	0
	3	200	0
	3	100[c]	0
	3	500	3
	3	250	3
	3	500[d]	0

continued next page

TABLE 10. (cont'd)

	3	200	3

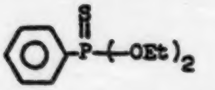
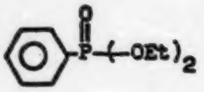
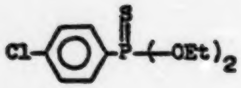
[a] Highest dose tested

[b] Showing any stage of ataxia

[c] This compound was tested both orally and intraperitoneally

[d] Required several doses of atropine to offset acute effects

TABLE 11. DELAYED NEUROTOXICITY OF O,O-DIETHYL ESTERS OF PHENYLPHOSPHONOTHIOATE, PHENYLPHOSPHONATE, AND 4-CHLOROPHENYLPHOSPHONOTHIOATE

Compound	Dose (mg/kg)	No. treated	Mean days after treatment when ataxia was observed T_1[a]	T_2	T_3	T_4
	500	3	-[b]	10	11	13
	300	3	-	12	13	15
	200	3	-	11	12	16
	150	3	-	-	10	14
	100	3	?	-	-	-
	250	3	-	-	10	13
	100	3	20	-	-	-
	200	3	11	12	15	18
	150	1	-	14	16	18
	125	1	-	-	-	-
	100	3	-	-	-	-

---

[a] T_1 = clumsy; T_2 = drunken; T_3 = sitting on hocks; T_4 = unable to stand.

[b] This stage of ataxia was not observed.

phenylphosphonothioate and phenylphosphonate is also of considerable interest since, in contrast to other neurotoxic organophosphorus esters, these compounds are not considered to be active inhibitors of esterases owing to their relatively inert nature.

Summary - The delayed neurotoxicity of technical and purified *O*-(4-bromo-2,5-dichlorophenyl) *O*-ethyl phenylphosphonothioate administered orally to hens was compared under different conditions. The technical material was neurotoxic at 750 mg/kg while the purified compound was neurotoxic at 1000-1250 mg/kg. The chemical composition of the technical material was analyzed for organophosphate contaminants. Impurities present as more than 0.1 percent were examined for delayed neurotoxic activity. *O,O*-Diethyl phenylphosphonothioate, its oxon analog, and *O,O*-diethyl 4-chlorophenylphosphonothioate were 5-10 times more potent as delayed neurotoxins than the parent compound. These impurities evidently potentiate the delayed neurotoxicity of ethyl leptophos and may potentiate the delayed neurotoxicity of other *O*-ethyl phenylphosphonothioate pesticides as well.

4. *References*.

1. Johnson, M.K., *CRC Crit. Rev. Toxicol.* *3*, 289 (1975).
2. Shea, K.P., *Environment* *16*, 6 (1974).
3. Abou-Donia, M.B., S.H. Preissig, *Toxicol. Appl. Pharmacol.* *35*, 269 (1976).
4. Abou-Donia, M.B., S.H. Preissig, *Toxicol. Appl. Pharmacol.* *38*, 595 (1976).
5. Sanborn, J.R., R.L. Metcalf, L.G. Hansen, *Pestic. Biochem. Physiol.* *7*, 142 (1977).
6. Ohkawa, H., N. Mikami, Y. Okuno, J. Miyamoto, *Bull. Environ. Contam. Toxicol.* *18*, 534 (1977).
7. Allahyari, R., P.W. Lee, G.H.Y. Lin, R.M. Wing, T.R. Fukuto, *J. Agric. Food Chem.* *25*, 471 (1977).
8. Lee, P.W., R. Allahyari, T.R. Fukuto, *Pestic. Biochem. Physiol.* *8*, 146 (1978).
9. Abou-Donia, M.B., D.G. Graham, *Toxicol. Appl. Pharmacol.* *45*, 685 (1978).
10. Abou-Donia, M.B., D.G. Graham, *Toxicol. Appl. Pharmacol.* *46*, 199 (1978).
11. Abou-Donia, M.B., D.G. Graham, *Toxicol. Appl. Pharmacol.* *48*, 57 (1979).

H. MODE OF ACTION OF PHOSPHORAMIDOTHIOATE ESTERS

Introduction - Although methamidophos (O,S-dimethyl phosphoramidothioate) is one of our most important insecticides and has been in agricultural use for a number of years, relatively little is known about its mechanism of action. For example, methamidophos is a relatively poor anticholinesterase, yet is highly toxic to animals which die of typical cholinergic symptoms of poisoning. Further, in its inhibition of acetylcholinesterase, it is not known whether the P-O or P-S linkage in methamidophos is cleaved during the inhibition process. This section is concerned with a study designed to determine the nature of the phosphorylating moiety when electric eel acetylcholinesterase (EEAChE) is inhibited by methamidophos.

Chromatography - It was necessary first to establish the chromatographic behavior of EEAChE and methamidophos in the Sephadex G-25 column. EEAChE (5 units) and 2-3 mg Blue Dextran, an indicator which chromatographs with high molecular weight proteins, were applied to the column and eluted with phosphate buffer. Enzymatic analysis of each fraction showed the enzyme to be associated with fractions 9-12. Blue Dextran also eluted with the enzyme fractions. This experiment was repeated 6 times and the void volume preceding elution of the enzyme was determined to be 4.0-4.5 mL.

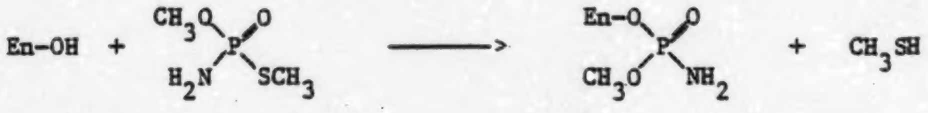
In analogous experiments, ^{14}C-methamidophos was observed to elute in fractions 26-31 with a total recovery of radioactivity of >98%. Analysis of the eluted radioactivity showed it to be in the form of unchanged methamidophos. These experiments established the feasibility of Sephadex G-25 in separating EEAChE from methamidophos.

Enzyme Inhibition - EEAChE was inhibited by either [O-methyl ^{14}C]- or [S-methyl ^{14}C]methamidophos and the reaction mixture was subjected to Sephadex chromatography. When [O-methyl ^{14}C]methamidophos was used, radioactivity was recovered in two distinct peaks as shown by the chromatographic profiles in Figure 1. Two different batches of enzyme were employed. The small peak, consisting of fractions 9-14 (peak A, 500 units enzyme) and fractions 12-17 (peak A', 1000 units enzyme), corresponds to EEAChE. The second peak, consisting of fractions 16-32 (peak B) and fractions 19-37 (peak B'), corresponds to methamidophos. Peak B' was approximately twice the size of B, in general agreement with the relative amounts of EEAChE employed in the separate experiments.

The fractions in peak A were each examined for hydrolysis of acetylthiocholine and found to have little or no enzymatic activity. Addition of 2-PAM to each fraction, followed by 30 min incubation, resulted in a substantial increase in enzymatic activity in each fraction. This provided additional evidence for the presence of the enzyme in peak A. Because of the low level of radioactivity in the fractions and the large dilution which takes place in Sephadex gel filtration, it was not possible to determine whether radioactivity was removed from the enzyme in the regeneration process.

Incubation of EEAChE (500 units) with excess [S-methyl ^{14}C]methamidophos, following by Sephadex chromatography resulted in the chromatographic profile shown in Figure 2. In this case, only one radioactive peak was observed (fractions 22-36), i.e., to the peak associated with methamidophos. As in the study with the O-methyl labeled material, treatment of each enzyme fraction with 2-PAM resulted in increased enzymatic activity in fractions 9-12, indicating the presence of EEAChE in these fractions.

Summary - The results described above provide strong evidence for P-S bond cleavage in the reaction leading to the inhibition of acetylcholinesterase by methamidophos. The inhibition reaction may be depicted as follows,



where, En-OH is the enzyme. In the inhibition reaction the serine hydroxyl group in the enzyme is phosphorylated by the amido(methoxy)-phosphinyl moiety of methamidophos with simultaneous loss of the thiomethyl moiety.

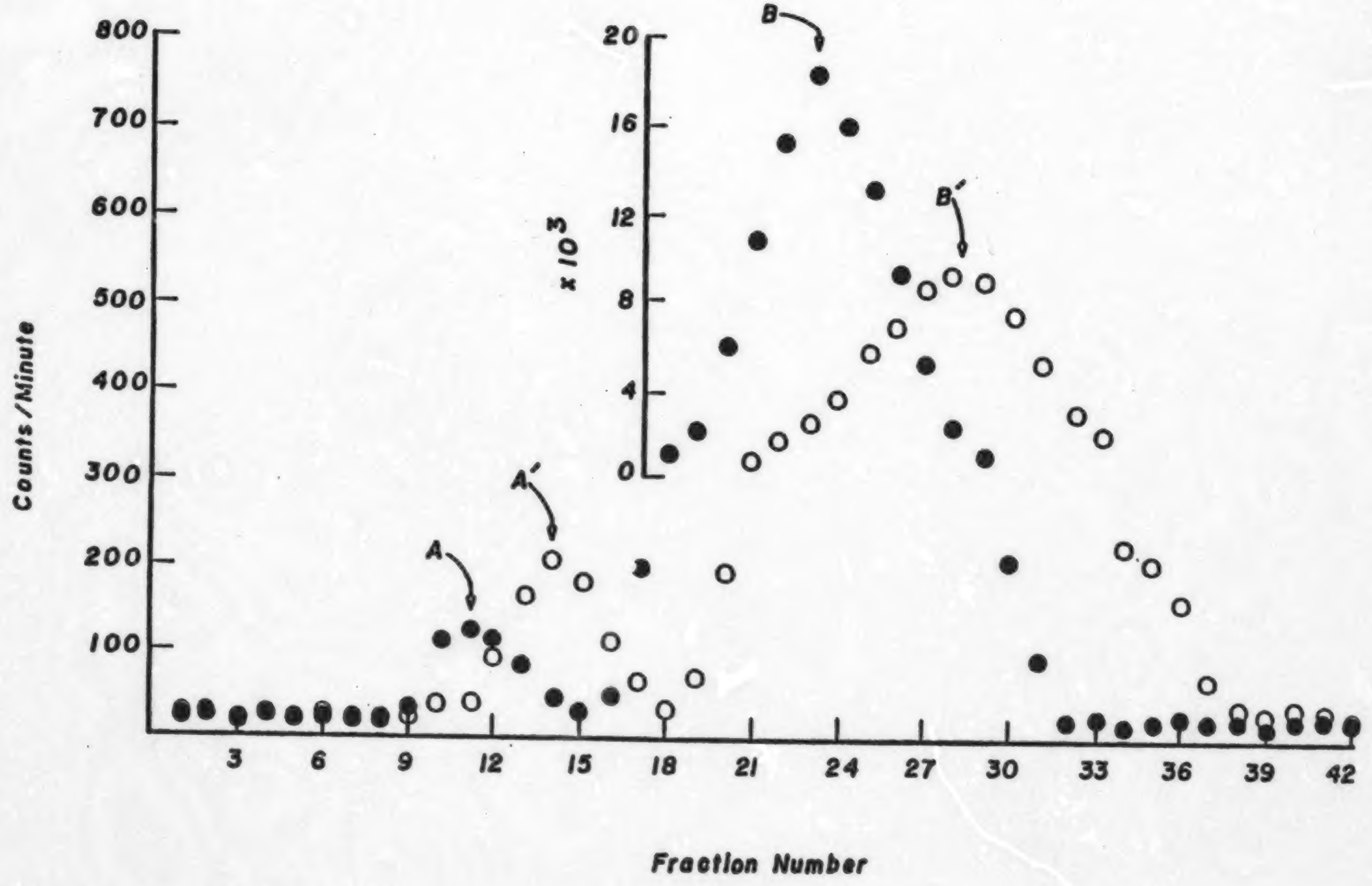


Figure 1. Plot of counts/minute vs relative fraction for $^{14}CH_3O)(CH_3S)P(O)NH_2$: (●—●) - 500 units EEAChE; (O—O) - 1000 units EEAChE.

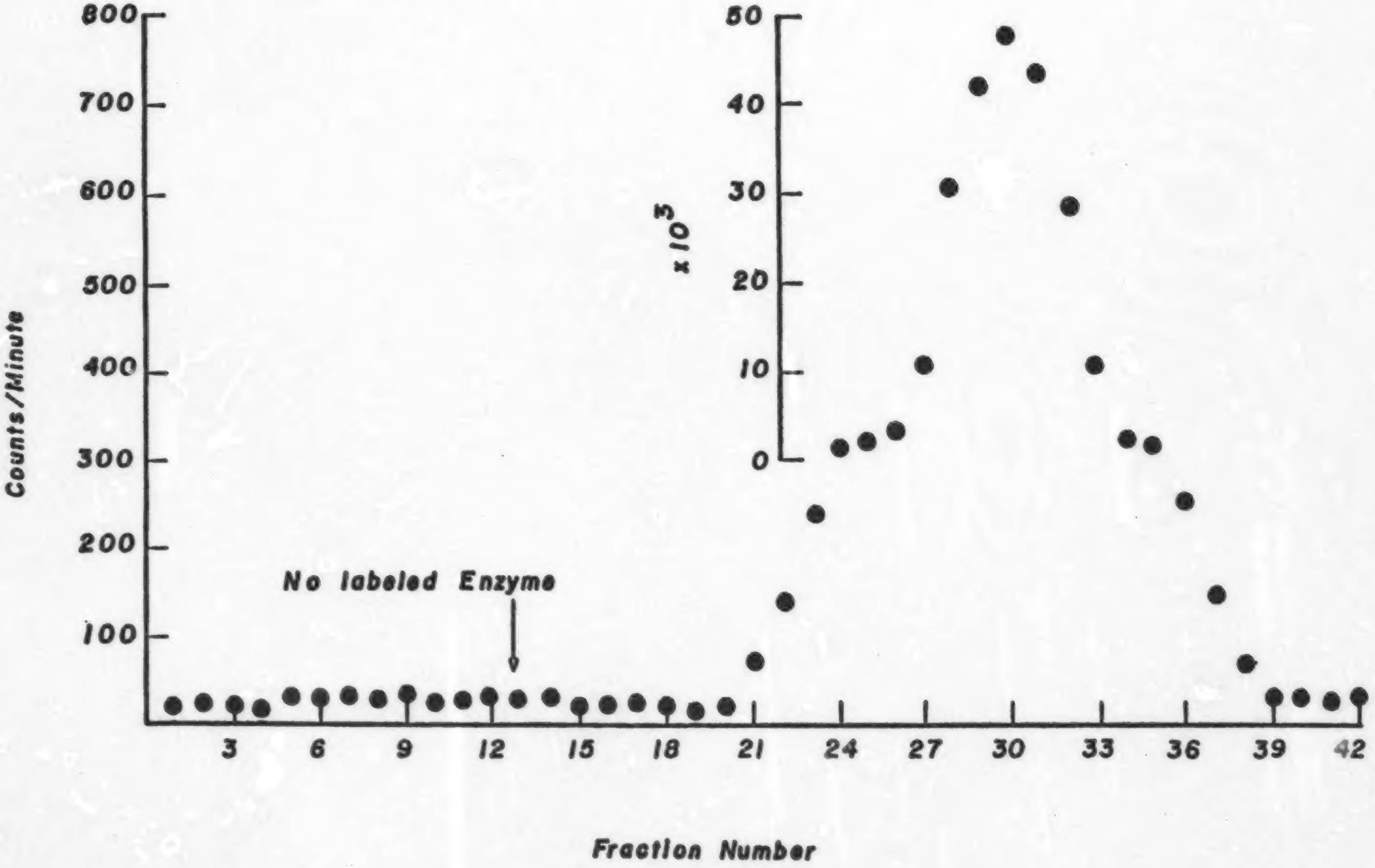


Figure 2. Plot of counts/minute vs relative fraction for $(CH_3O)(^{14}CH_3S)P(O)NH_2$: (●—●) - 500 units of EEAChE.

I. GAS CHROMATOGRAPHY OF INSECTICIDES

Introduction - Direct gas chromatography of some insecticides, particularly most carbamates and many phosphates, has presented difficulties because of on column pyrolysis or retention on active sites. Various investigators have been actively searching for ways to alleviate these difficulties with limited success. Recently the development of a novel gas chromatographic column (surface-modified support), obtained by high temperature conditioning and subsequent exhaustive methanol extraction of 6% Carbowax 20M on HCl-extracted Chromosorb W, was reported[1]. This new column exhibited the more desirable features of both gas-solid and gas-liquid chromatography - low bleed, short retention times at relatively low temperatures, excellent peak symmetry and resolution of highly polar compounds, and high surface inactivation with little indication of decomposition of labile compounds. Further studies have demonstrated that the methodology is not limited to Carbowax 20M as a stationary phase[2]. The potential of the surface-modified Carbowax 20M support for the direct gas chromatography of carbamate insecticides has been shown[3], but this novel methodology has not been followed up further with insecticides or extended to other pesticides and the evaluation of other support phases for the gas chromatography of pesticides.

Organophosphorus Insecticides - We have evaluated over 40 stationary phases as surface-modified supports. Although most of the columns show some promise for phosphorothionates, relatively few show broad promise for phosphates and even fewer for carbamates. It is of interest that the columns which are most effective for phosphates and carbamates fall in a similar limited range of intermediate polarities based on McReynold's constants. In most previous studies, non-polar phases on silanized supports were more successfully used.

Retention times for the gas chromatography of 16 organophosphorus insecticides on the most effective or interesting columns are shown in Table 1. The most effective columns for both phosphorothionates and phosphates were prepared with AN-600, Reoplex 400, and EGSP-Z. The SILAR-9CP column is also of interest because it shows the greatest retention differences between phosphorothionates and their oxygen analogs. Although the Carbowax columns exhibit excellent chromatography of individual compounds, the lack of resolution of most phosphorothionates and their oxygen analogs limits their usefulness. A specific example of the potential usefulness of the AN-600, Reoplex 400, and EGSP-Z columns is shown in Table 2 for the resolution of fenthion, fenoxon, and their sulfoxides and sulfones at a single isothermal temperature. Although these columns provide new possibilities for gas chromatography of phosphates and resolution of phosphorothionates and their oxygen analogs, we have still been unsuccessful in chromatographing all phosphates including guthion oxon [O,O-dimethyl S-(4-oxo-1,2,3-benzotriazin-3(4H)-ylmethyl)phosphorothionate] or O-methyl O-2,4-dichlorophenyl phosphoramidate for example on any of the columns.

TABLE 1. GAS CHROMATOGRAPHY OF ORGANOPHOSPHORUS INSECTICIDES ON SURFACE-MODIFIED SUPPORTS.

Retention times in seconds under the following experimental conditions: column 6 feet x 2 mm i.d. (except for Reoplex 400 4 feet x 2 mm); column temperature 185°C; gas flows for hydrogen, helium and air 40, 27 and 320 ml/min, respectively; and detector AFID (KCl).

	Retention Time in Seconds with Following Stationary Phases					
OP Compound	An-600	Reoplex 400	EGSP-Z	Silar-9CP	Carbowax 20M	Carbowax 1540
1. fonofos	71	29	29	37	57	30
2. fenthion	155	98	105	74	133	63
3. fenoxon	209	120	130	350	158	81
4. Chlorthion	301	152	148	98	192	97
5. Chloroxon	367	175	169	269	218	103
6. methamidophos	59	28	36	102	53	32
7. acephate	132	54	67	210	85	45
8. parathion	200	119	120	118	154	82
9. paraoxon	240	130	133	408	158	81
10. dimethoate	200	109	109	170	151	76
11. dimethoate oxon	217	88	106	390	124	62
12. carbophenothion	360	284	296	168	373	182
13. crufomate	290	207	212	1000	287	150
14. fensulfothion	870	573	600	1050	749	300
15. leptophos	620	462	519	128	664	279
16. leptophos oxon	690	494	554	Ca.1000	670	280

TABLE 2. GAS CHROMATOGRAPHY OF FENTHION, FENOXON, AND THEIR SULFOXIDES AND SULFONES ON SURFACE-MODIFIED SUPPORTS.

Retention times in seconds at a column temperature of 200°C and same other experimental conditions used for organophosphorus insecticides as shown in Table 1.

	Retention Time in Seconds with Following Stationary Phases		
OP Compound	An-600	Reoplex 400	EGSP-Z
fenthion	97	62	69
fenthion sulfoxide	670	342	410
fenthion sulfone	890	444	467
fenoxon	127	72	81
fenoxon sulfoxide	790	406	526
fenoxon sulfone	1000	518	575

EGSP-Z and NPGS were selected as stationary phases for the comparison of surface-modified supports with normal low-loaded supports for the direct gas chromatography of organophosphates. The low-loaded supports were prepared at 2.5% by weight on chromosorb 750, 80/100 mesh, in a comparable manner to the surface-modified supports except for high temperature conditioning and exhaustive solvent extraction. The results of the chromatography of seventeen organophosphates on columns prepared from these supports are shown in Table 3. It is obvious from these results that the surface-modified supports successfully chromatograph more of the compounds and at lower temperatures than do the low-loaded supports. In addition, the resolution of the surface-modified supports is better than that of the low-loaded supports as indicated by the data in the table for phosphorothionates and their oxons. Of special interest is the successful chromatography of azinphosmethyl oxon on the NPGS surface-modified support.

One of the problems in analysis of organophosphates has been adequate resolution of the parent phosphorothionate and its oxon in order to determine the oxon quantitatively in the presence of the phosphorothionate in amounts a number of orders of magnitude greater. It had been previously determined that the Silar-9CP surface-modified support provided excellent resolution of phosphorothionates and their oxons, although the much later eluting oxons had a tendency to tail. It was of interest, therefore, to determine if mixtures of Silar-9CP and NPGS surface-modified supports would retain the resolution characteristic of Silar-9CP as well as the better peak shape and chromatographability of difficult compounds, such as azinphosmethyl oxon, of NPGS. Mixtures of previously prepared NPGS and Silar-9CP surface-modified supports of 1:2 and 1:1 by weight were prepared by fluidizing to obtain uniform mixing. The results of chromatographing seventeen organophosphates on columns of various lengths prepared from these mixtures are shown in Table 4. These data suggest that there are excellent possibilities for achieving a desired level of resolution by the use of such mixtures.

Direct Gas Chromatography of Carbamate Insecticides - Direct gas chromatography of carbamate pesticides has presented difficulties because of on column pyrolysis or retention on active sites. Various investigators have been actively searching for ways to alleviate these difficulties with limited success[2,3]. Most recently Hall and Harris[4] reported the successful chromatography of 24 carbamates on a commercially available surface-modified carbowax 20M support (Ultra-Bond, RFR, Hope, RI and Ultra-Bond coated with 3% OV101, 1% OV17, 1% OV210, 1% Carbowax 20M, or 0.5% OV210 + 0.65% OV17. However, unsatisfactory results were obtained for aldicarb, aldicarb sulfoxide, aldicarb sulfone, Karbutylate, methomyl, phenmedipham and thiophanate. Carbaryl, which had a long retention time, exhibited considerable on column degradation. They concluded that moderate column temperatures (<185°C) and relatively short analysis times were critically important.

TABLE 3. COMPARISON OF EGSP-Z AND NPGS PREPARED AS SURFACE-MODIFIED AND NORMAL LOW-LOADED (2.5%) SUPPORTS FOR THE GAS CHROMATOGRAPHY OF ORGANOPHOSPHORUS INSECTICIDES

Experimental Conditions: Hewlett-Packard 402 gas chromatograph; column for EGSP-Z 6 feet X 2 mm, for NPGS 4 feet X 2 mm; gas flows for hydrogen, helium, and air 23, 37, and 266 ml/min, respectively; AFID (Rb_2SO_4) detector.

	OP Compound	Retention Time in Seconds with Following Stationary Phases and Conditions					
		EGSP-Z			NPGS		
		Surface-Modified	Low-Loaded		Surface-Modified	Low-Loaded	
		185°	185°	230°	185°	185°	220°
1	Fonofos	43	160	48	31	90	32
2	Methamidophos	44	78	28	30	75	25
3	Acephate	78	201	57	61	NDP	NDP
4	Fenthion	105	528	117	84	306	84
5	Fenoxon	130	587	124	100	NDP	NDP
6	Chlorthion	148	729	151	148	NDP	NDP
7	Chloroxon	169	805	154	170	NDP	NDP
8	Parathion	120	534	111	109	393	110
9	Paraoxon	133	572	116	122	NDP	NDP
10	Dimethoate	109	472	103	98	NDP	NDP
11	Dimethoate oxon	106	331	80	89	NDP	NDP
12	Crufomate	212	812	149	180	628	137
13	Carbophenothion	296	1532	264	247	906	210
14	Methamidathion	358	1277	231	182	NDP	NDP
15	Methamidaoxon	466	NDP	Decomposes	214	NDP	NDP
16	Azinphosmethyl	>1200	-	>1200	1410	-	>1500
17	Azinphosmethyl oxon	NDP[1/]	-	NDP	1643	-	NDP

[1/] NDP = No detectable peak

TABLE 4. GAS CHROMATOGRAPHY OF ORGANOPHOSPHATES ON MIXTURES OF NPGS AND SILAR-9CP SURFACE-MODIFIED SUPPORTS

Experimental Conditions: Hewlett-Packard 402 gas chromatograph; column X (as shown below) feet X 2 mm; gas flows for hydrogen, helium, and air 23, 37, and 266 ml/min, respectively; AFID (Rb_2SO_4) detector; oven temperature 185°C.

	OP Compound	Retention Time in Seconds with Following Ratios of NPGS and Silar 9CP Surface-Modified Supports by Weight.				
		1:2	1:1			
		6 ft	6 ft	4 ft	3 ft	2 ft
1	Fonofos	23	40	24	15	-
2	Methamidophos	54	62	35	22	-
3	Acephate	-	116	69	45	-
4	Fenthion	50	85	55	37	-
5	Fenoxon	129	182	101	66	-
6	Chlorthion	70	140	85	58	-
7	Chloroxon	121	203	115	79	-
8	Parathion	62	120	73	50	-
9	Paraoxon	136	199	105	72	-
10	Dimethoate	83	127	72	50	-
11	Dimethoate oxon	174	209	98	66	-
12	Crufomate	380	235	224	153	-
13	Carbophenothion	118	468	142	98	-
14	Methamidathion	-	183	112	75	-
15	Methamidaoxon	-	387	207	138	-
16	Azinphosmethyl	574	1220	738	518	285
17	Azinphosmethyl oxon	NDP[1/]	NDP	1143	788	513

[1/] NDP = No detectable peak

We have evaluated surface-modified supports for direct chromatography of carbamate insecticides and the temperature-programmed separation of impurities in their technical products. These studies have been materially aided by the acquisition of a Varian 3700 gas chromatograph with a thermionic specific detector (TSD) providing ready detection sensitivity for carbamates and their N-containing impurities in the 1-10 ng range.

Twelve carbamate insecticides of a variety of chemical structures and physical properties were evaluated. Our most successful results have been achieved with a 2 ft x 2 mm I.D. Nickel 200 column containing a surface modified Carbowax 1540 support. Isothermal chromatography was carried out at column temperatures from 100°-130°C and programmed-temperature chromatography was carried out from 50°-130°C at 10°/min. Injector temperature was very critical. The most effective injector temperature was 110°; lower temperatures did not provide adequate initial volatilization for sharp symmetrical peaks and higher temperature promoted on column pyrolysis. Carrier gas (He) flow was 35 ml/min and hydrogen and air were optimized for the TSD at 5 and 175 ml/min, respectively.

Retention times for 10 carbamates at three column temperatures (110°, 120° and 130°C) are shown in Table 5 and representative isothermal chromatograms of aldicarb (10 ng), Pyrolan (10 ng) and carbaryl (50 ng) at 130°C are shown in Fig. 1A and 1B. Note the narrow symmetrical peak for the more volatile aldicarb but the broader, slightly tailing peaks for the less volatile pyrolan and carbaryl. Pyrolan is sufficiently stable to permit peak improvement at higher column

TABLE 5. GAS CHROMATOGRAPHY OF CARBAMATE INSECTICIDES ON A SURFACE-MODIFIED CARBOWAX 1540 SUPPORT.

Carbamate	Retention Times in Seconds at Following Column Temperatures 110°	120°	130°C
1. Isolan	83	48	33
	95	55	
2. Aldicarb	190	101	64
3. 11203	225	118	74
4. MIPC	277	150	92
5. Propoxur	350	183	111
6. Methomyl	-	-	-
7. Carbofuran	782	394	232
8. Mexacarbate	900	471	278
9. 11395	-	-	-
10. 9699	1691	705	405
11. Pyrolan	1115	583	362
12. Carbaryl	2006	1119	635

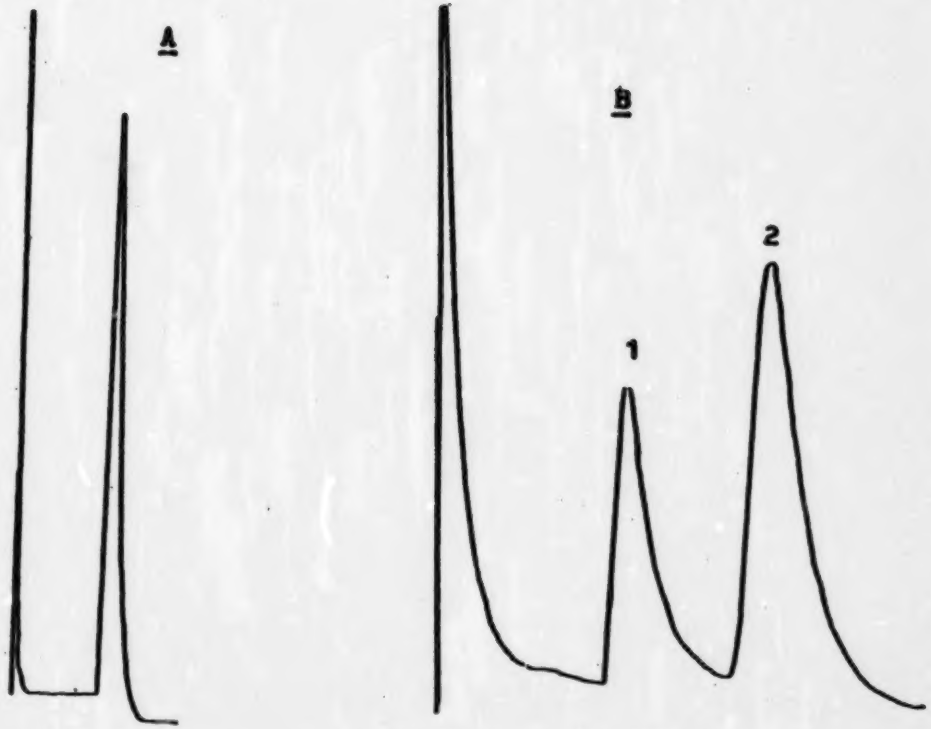


Figure 1. Chromatograms of aldicarb (A, 10 ng), Pyrolan (B1, 10 ng), and carbaryl (B2, 50 ng) at isothermal column temperature of 130°C and sensitivity of 10^{-12} x 16.

temperatures but carbaryl shows increasing on-column pyrolysis as temperatures are increased. Isothermal chromatography of methomyl and 11395 was unsuccessful at the 10 ng level due to inadequate volatilization and/or on-column pyrolysis.

Eleven surface-modified supports prepared with the following stationary phases: Carbowax 1500, 1540, 6000, and 20M, butanediol succinate (BDS), neopentyl glycol succinate (NPGS), STAP (steroid analysis phase), Reoplex 400, UCON 75-H-90M, EGSP-Z (ethylenesuccinate-phenylsilicone copolymer) and Triton X-305 were evaluated as 2 mm id x 61 cm Ni 200 columns. The isothermal and temperature-programmed chromatographic behavior of 12 carbamates was evaluated, 2 N,N-dimethyl carbamates: Isolan and Pyrolan; 7 N-methyl carbamates: MIPC (m-isopropylphenyl N-methyl carbamate), propoxur, bendiocarb, carbofuran, mexacarbate, Cmpd 9699 (O-propargylphenyl N-methyl-carbamate) and carbaryl; and 3 oxime carbamates: aldicarb, Cmpd 11203 (2-methyl-1-(methylthio)propionaldehyde-O-(methylcarbamoyl)oxime) and methomyl.

Based on evaluation of chromatographic behavior following injection of a mixture of carbamates at various isothermal column temperatures from 100-150°, surface-modified supports prepared with Triton X-305 and EGSP-Z provided the best separation with symmetrical narrow peaks and least decomposition of the oxime carbamates (see Fig. 2). The Carbowax and UCON 75H 90M columns provided reasonably good separation with symmetrical peaks but showed serious decomposition of the oxime carbamates. The STAP, NPGS, Reoplex 400, and BDS columns exhibited much poorer separation characteristics with wider though symmetrical peaks.

Composite chromatograms of the oxime carbamates, aldicarb and methomyl, at various isothermal column temperatures in Fig. 3 illustrate the relatively narrow temperature window for successful direct carbamate chromatography necessary to provide a high enough temperature for adequate volatilization but a low enough temperature to avoid decomposition.

Temperature-programmed chromatograms of aldicarb, propoxur, and methomyl in Fig. 4 indicate the potential use of these columns for separation of the components of technical carbamate insecticides. The fractionally-crystallized technical carbamates are much cleaner products than the previously studied technical organophosphorus insecticides. Chromatograms A (aldicarb) and C (methomyl) show the tendency of the oxime carbamates to decompose after 8 min on-column and column temperature rising above 100°. Chromatogram B of the less susceptible propoxur suggests potential promise for the separation and identification of the small number of impurities in this technical product.

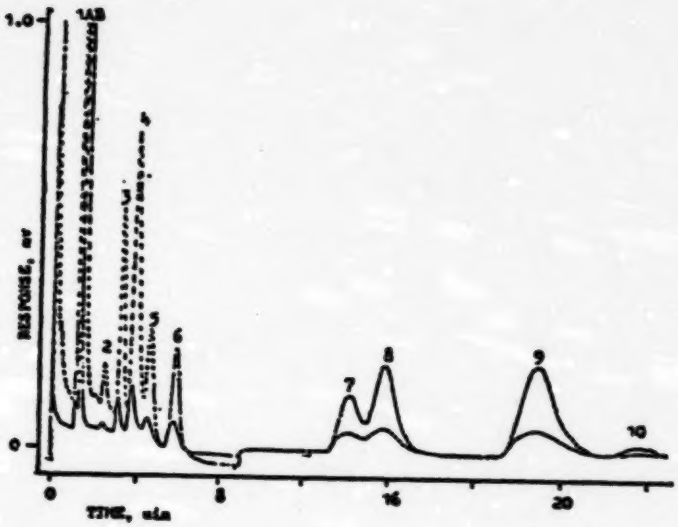


Fig. 2. Chromatogram of carbamate insecticides separated on a Triton X-305-modified support at isothermal temperature of 110°. Sensitivity: 8 (dotted line) and 64 (solid line) x 10^{-11} amp/mv. Compounds in order of elution are: 1A,B, Isolan; 2, mexacarbate phenol; 3, aldicarb; 4, Cmpd 11203; 5, MIPC; 6, propoxur; 7, carbofuran; 8, mexacarbate; 9, Pyrolan and 10, Cmpd 9699. Sample quantity: 100 ng each. Thermionic specific detector.

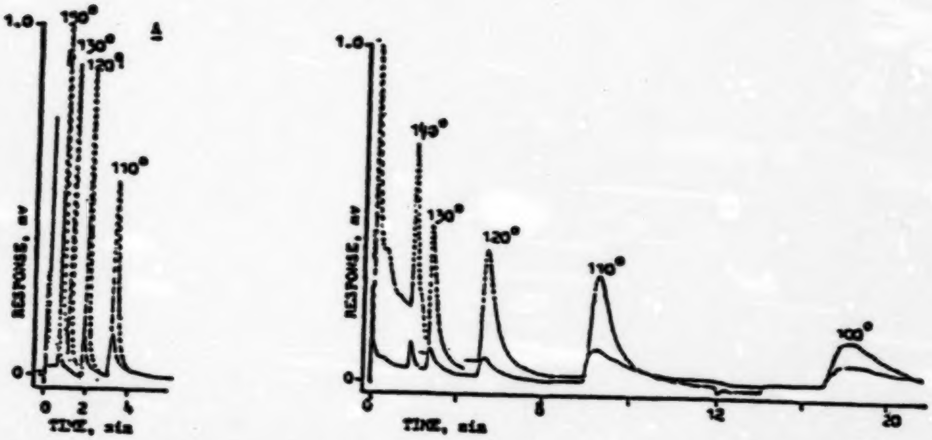


Fig. 3. Composite chromatograms of aldicarb (A) and methomyl (B) on a Triton X-305-modified support at various isothermal temperatures. Sensitivity: 8 (dotted line) and 64 (solid line) x 10^{-11} amp/mv. Sample quantity: 100 ng each. Thermionic specific detector.

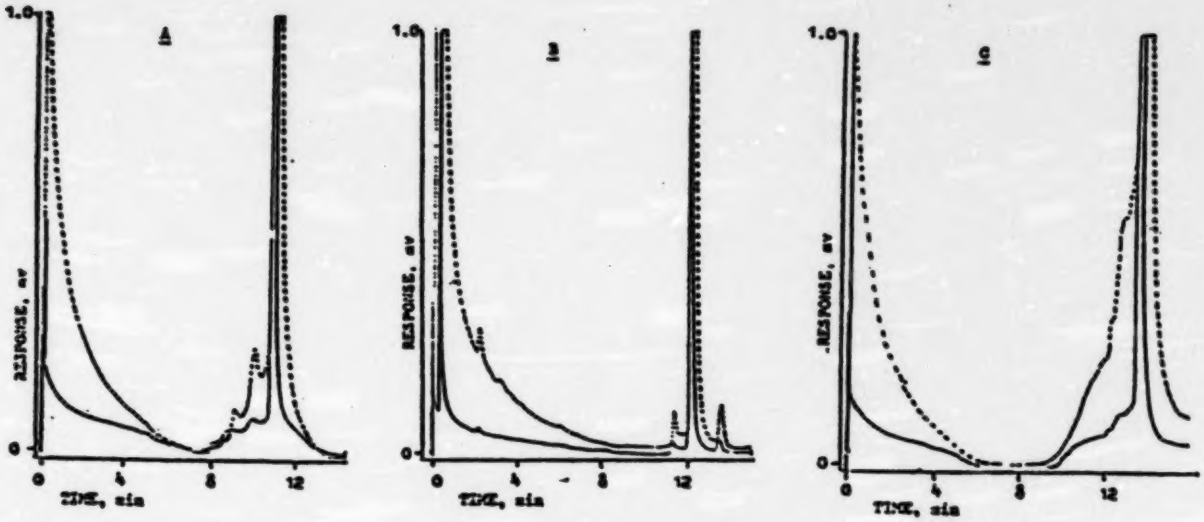


Fig. 4. Chromatograms of aldicarb (A), propoxur (B) and methomyl (C) on a Triton X-305-modified support, temperature programmed after 1 min delay from 50° to 230° at 7°/min. Sensitivity: 8 (dotted line) and 64 (solid line) x 10^{-11} amp/mv. Sample quantity: 1 µg each. Flame ionization detector.

Summary - The forty-four stationary phases prepared as surface-modified supports and evaluated over the past few years included: Apiezon L, SE-30, DC-200, OV-101, UCON LB-550-X, UCON LB-1200-X, Versamid 900, Poly MPE, Poly A 103, UCON 50-HB-280-X, QF1, UCON 75-H-90M, Triton X-305, HI-EFF-8-BP, EGSP-Z, Carbowax 20M, Carbowax 20MTPA, Carbowax 6000, Reoplex 400, Carbowax 1540, EGSS-Y, Hyprose SP-80, EGSP-A, SILAR-7CP, ECNSS-M, ECNSS-S, EGSS-X, SILAR-9CP, SILAR-10C, OV-275 (Applied Science Laboratories, State College, PA, 16801); OV-3, Dexsil 300, OV-17, AN-600, NPGS, Igepal CO-990, Epon Resin 1001, Carbowax 1500, DEGS (Analabs, Inc. North Haven, CT 06473), OV-210, OV-225 (Pierce Chemical Co., Rockford, IL 61105); BDS, Dexsil 400, and Ultra-Bond® PEGS (RFR Corp., Hope, RI 02831).

Several of these surface-modified supports exhibit excellent characteristics for the direct gas chromatography of organophosphorus and carbamate insecticides; e.g., low bleed, very good peak symmetry and resolution, short retention times at relatively low temperatures, and high surface inactivation with little decomposition of labile compound.

The most effective surface-modified supports for both phosphorothionates and phosphates including: fonofos, fenthion, fenoxon, Chlorthion, Chloroxon, methamidophos, acephate, parathion, paraoxon, dimethoate, dimethoate oxon, carbophenothion, methidathion, methidaoxon, crufomate, fensulfothion, leptophos, leptophos oxon, azinphosmethyl, and azinphosmethyl oxon, were prepared with AN 600, Reoplex 400, SILAR-9CP, NPGS, EGSP-Z, and BDS. These columns, for example, are effective in resolving fenthion, fenoxon, and their sulfoxides and sulfones at a single isothermal temperature. The SILAR-9CP column is of interest because it shows the greatest retention differences between phosphorothionates and their oxygen analogs. By physical mixing of the SILAR-9CP surface-modified support with such supports prepared with other phases in various proportions it is also possible to vary these retention differences. The NPGS surface-modified support is the only support that we have so far examined which successfully chromatographs azinphosmethyl oxon which pyrolyzes on-column above 205°C but requires an oven temperature of at least 185°C for volatilization.

Surface-modified supports have been evaluated for the direct gas chromatography of 12 carbamate insecticides of diverse chemical structures and physical properties and the temperature-programmed separation of impurities in their technical products. Promising results were achieved with a 2 ft x 2 mm I.D. Nickel 200 column containing a surface-modified Carbowax 1540 support. Effective isothermal separation of Isolan, aldicarb, 11203, MIPC, and propoxur with sharp symmetrical peaks were obtained at column temperatures of 100°-130°C and injection temperature of 110°C, less volatile carbamates carbofuran, mexacarbate, 9699, Pyrolan and carbaryl produced broader, less symmetrical peaks at these temperatures but, except for carbaryl which showed increased pyrolysis, higher column temperatures appear promising for improving peak shape and symmetry. Temperature-programmed chromatography of the technical insecticides from 50° to 130°C at 10°/min appear promising for the separation of impurities for further identification studies. Triton X-305 and EGSP-Z also showed promise for the direct chromatography of a number of carbamate insecticides because of excellent separation characteristics on short columns, providing for short retention times, at low enough temperatures to avoid major on-column decomposition but high enough to provide for adequate volatilization.

REFERENCES

1. Aue, W. B., C. R. Hastings, S. Kapila, J. Chromatog. 77, 299(1973)

2. Aue, W. B., C. R. Hastings, K. O. Gerhardt, J. Chromatog. 99, 45(1974)

3. Lorah, E. J., D. D. Hemphil, J. Assoc. Off. Agr. Chem. 57, 570(1974)

4. Hall, R. C., D. E. Harris, J. Chromatog. 169, 245(1979)

J. NEUROPHYSIOLOGICAL STUDIES ON THE MODE OF ACTION OF INSECTICIDES

The pyrethroid insecticides were found to poison the nervous system by acting at specific sites on neurons. On motor nerves, all poisoning was confined to presynaptic nerve terminals; in sensory nerves the sensory endings were poisoned. During pyrethroid poisoning, discharges of nerve impulses propagated both antidromically and orthodromically from the terminal. Although the central nervous system was also poisoned, the subcellular sites involved were not investigated at the neuron level.

Picrotoxinin, a natural plant convulsant, was toxic to the desheathed housefly central nervous system (CNS), and was much less toxic to the intact CNS. This pointed to an effective detoxication or other protective mechanism inherent in the perineurial sheath against picrotoxinin. It also suggested that the picrotoxinin structure could serve as a model for synthesis of a new category of insecticide if the cause of the protective mechanism were found and compounds synthesized to overcome it.

Despite varying widely in lipophilicity, a series of substituted *N*,*N*'-thiodicarbamates were found to poison intact houseflies with the *same* latency from application to disruption of flight motor neuron coordination. Evidence suggested that the sulfenylated carbamate analogs were converted very rapidly at the cuticle to the parent carbamate and that this conversion was not rate-limiting. The equimolar toxicity of the *N*,*N*'-thiodicarbamate analogs and their parent compounds can thus be understood. This finding also, more importantly, provides a new synthesis strategy for delivering toxic chemicals to the inside of the insect.

A housefly strain was obtained which contained the kdr factor which rendered the strain resistant to pyrethroid and DDT insecticides. It was shown that the entire nervous system of the kdr houseflies was resistant to direct perfusion of pyrethroids and DDT. Both the central and peripheral nervous systems were refractory to compounds. In addition, the perineurial sheath on the central nervous system made little difference to bioassay responses whether intact or removed, and therefore, played no role in the resistance (it might be noted, as mentioned above, that the sheath of susceptible houseflies protects the CNS against picrotoxinin). This demonstrated that the nervous system of the kdr strain of housefly is site-insensitive to pyrethroids. An experimental procedure was outlined which measures quantitatively the amount of kdr present in mosquitoes and houseflies.

In the process of investigating the actions of insecticides on motor nerve terminals, it was found that the 'slow' and 'fast' motor units of Dipteran maggots are mediated by different neurotransmitters; that aspartic acid mimics the 'slow' neurotransmitter and glutamic acid the 'fast' neurotransmitter. However, a major problem was encountered with accepting any amino acid as a neurotransmitter when it was shown that normal, neurallyevoked synaptic transmission occurred in the presence of slightly elevated calcium concentrations, which also rendered the preparations completely insensitive to perfused or iontophoretically applied asparate or glutamate under the same conditions. The later discrepancy represents a major problem in understanding insect skeletal neuromuscular transmission.

Attempts were made at distinguishing between DDT and pyrethroid actions on the insect nervous system. A list of criteria were reported which allowed analogs to be categorized as DDT-like or pyrethroid-like.

The consequences of insecticide poisoning were investigated by comparing the histological appearance of the thoracic ganglion of houseflies which had been treated with doses causing mortality in 50% of a population. Lindane treatment was found to cause "vacuoles" in the thoracic ganglion which were correlated in number with the amount of disability as measured behaviorally. The type of disability was correlated with the site of vacuolization.

All too often, actions of insecticides on cockroach or crayfish nerves are compared in tables and graphs with toxicity of the same compound on houseflies because the vast majority of toxicity data comes from the housefly. Such comparisons are questionable and do not stand close examination.

We pioneered the detailed examination of activity of the central nervous system of the housefly by recording and comparing motor unit activity before, during, and after poisoning. These techniques were possible without dissection and during performance of uninhibited movement, i.e., cleaning, walking, and flying, with the only restriction being a tether and wires inserted through the cuticle in a few sites on the thorax. This approach allowed the first unambiguous description of overt poisoning symptoms based on nervous activity.

By combining this recording method with various types of stimulation, we have been able to excite known reflex pathways and so, monitor sensory, interneuronal and motor neuronal activity of identified neurons during poisoning. This technique has lead to a distinction between knockdown caused by actions on peripheral nerves versus knockdown caused by actions on the central nervous system.

Because of these experimental approaches, we know far more about the actions of insecticides on insects than ever before, and we are now able to concentrate on specific parts of the poisoning process, e.g., early knockdown and reversible actions versus long-term and irreversible consequences of poisoning. Without using neurophysiological methods, it would be impossible to determine what neurotoxic insecticides are doing to the insect nervous system, because the only method of assessing neurotoxic actions is to define the functional impairment.

LIST OF INVENTIONS OR PUBLICATIONS

1. Krieger, R. I., P. W. Lee, M. A. H. Fahmy, M. Chen, and T. R. Fukuto. 1976. Metabolism of 2,2-dimethyl-2,3-dihydrobenzofuranyl-7 N-dimethoxyphosphinothioyl-N-methylcarbamate in the house fly, rat and mouse. Pestic. Biochem. Physiol. 6: 1-9.

2. Lee, P. W., and T. R. Fukuto. 1976. Penetration and comparative metabolism of leptophos in susceptible and resistant house flies. Arch. Environ. Contam. Toxicol. 4: 443-455.

3. Takade, D. Y., M. S. Seo, and T. R. Fukuto. 1976. Alteration of O,O-dimethyl S-[α-(carboethoxy)benzyl] phosphorodithioate (phenthoate) in citrus, water, and upon exposure to air and sunlight. Arch. Environ. Contam. Toxicol. 5: 63-86.

4. Rajakovick, A. S. and R. B. March. 1976. Insecticide cyclic nucleotide interactions I. Quinoxalinedithiol derivatives: a new group of potent phosphodiesterase inhibitors. Pestic. Biochem. Physiol. 6: 10-19.

5. Miller, T. 1976. Distinguishing between carbamate and organophosphate insecticide poisoning in house flies by symptomology. Pestic. Biochem. Physiol. 6: 307-19.

6. Takade, D. Y., T. Allsup, A. Khasawinah, and T. R. Fukuto. 1976. Metabolism of O,O-dimethyl S-[α-(carboethoxy)benzyl] phosphorodithioate (phenthoate) in the white mouse and house flies. Pestic. Biochem. Physiol. 6: 367-376.

7. Desmarchelier, J. M., D. A. Wustner, and T. R. Fukuto. 1976. Mass spectra of organophosphorus esters and their alteration products. Res. Revs. 63: 76-185.

8. Krieger, R. I., A. S. Salhab, P. W. Lee, and T. R. Fukuto. 1976. Absorption and metabolism of a selective insecticide, 2,2-dimethyl-2,3-dihydrobenzofuranyl-7 N-dimethoxyphosphinothioyl-N-methylcarbamate, in bean plants. Arch. Environ. Contam. Toxicol. 4: 483-491.

9. Lee, P. W., R. Allahyari, and T. R. Fukuto. 1976. Stereospecificity in the metabolism of the chiral isomers of fonofos by mouse liver microsomal mixed function oxidase. Biochem. Pharmacol. 26: 2671-2674.

10. Kao, T. S., and T. R. Fukuto. 1977. Metabolism of O,S-dimethyl propionyl- and hexanoylphosphoramidothioate in the house fly and white mouse. Pestic. Biochem. Physiol. 83-95.

11. Miller, T. and M. E. Adams. 1977. Central vs. pheripheral action of pyrethroids on the housefly nervous system. In: Synthetic Pyrethroids, Ed. by M. Elliott, ACS Symposium Series, No. 42, pp. 98-115.

12. Allahyari, R., P. W. Lee, G. H. Y. Lin, R. M. Wing, and T. R. Fukuto. 1977. Resolution and reactions of the chiral isomers of O-ethyl S-phenyl ethylphosphonodithioate (fonofos) and its analogues. J. Agr. Food Chem. 25: 471-478.

13. Umetsu, N., F. H. Grose, R. Allahyari, S. Abu-El-Haj, and T. R. Fukuto. 1977. The effect of impurities on the mammalian toxicity of technical malation and acephate. J. Agr. Food Chem. 25: 946-953.

14. Fukuto, T. R. 1977. Approaches to selective toxicity. J. Pestic. Sci. 2: 541-548.

15. Lee, P. W., R. Allahyari, and T. R. Fukuto. 1978. Studies on the chiral isomers of fonofos and fonofos oxon. I. Toxicity and anti-esterase activities. Pestic. Biochem. Physiol. 8: 146-157.

16. Lee, P. W., R. Allahyari, and T. R. Fukuto. 1978. Studies on the chiral isomers of fonofos and fonofos oxon. II. In vitro metabolism. Pestic. Biochem. Physiol. 8: 158-169.

17. Lee, P. W., R. Allahyari, and T. R. Fukuto. 1978. Studies on the chiral isomers of fonofos and fonofos oxon. III. In vivo metabolism. Pestic. Biochem. Physiol. 9: 23-32.

18. Fahmy, M. A. H., N. M. Mallipudi, and T. R. Fukuto. 1978. Selective toxicity of N,N'-thiodicarbamates. J. Agric. Food Chem. 26: 550-557.

19. Khasawinah, A. H. A., R. B. March, and T. R. Fukuto. 1978. Insecticidal properties, antiesterase activities, and metabolism of methamidophos. Pestic. Biochem. Physiol. 9: 211-221.

20. Wustner, D. A., Cecil Smith, and T. R. Fukuto. 1978. Aryl N-methoxy-N-methylcarbamates, potent competitive reversible inhibitors of cholinesteases. Pestic. Biochem. Physiol. 9: 281-292.

21. Fukuto, T. R. 1978. Insecticide metabolism and mode of action. 26th International Congress of Pure and Applied Chemistry. Pure & Appl. Chem. 50: 1015-1032.

22. Zerba, E., and T. R. Fukuto. 1978. Hydrolytic and toxicological properties of ethyl α-cyanobenzaldoxime phosphoramidates. J. Agric. Food Chem. 26: 1365-1369.

23. Wustner, D. A., H. T. Reynolds, and T. R. Fukuto. 1978. Insecticidal properties of phosphonamidothioate esters and derivatives. J. Agric. Food Chem. 26: 1104-1107.

24. Fukuto, T. R. 1978. Effect of structure on the interaction of organophosphorus and carbamate esters with acetylcholinesterase. *In*: Neurotoxicology of Insecticides and Pheromones. T. Narahashi, Ed., Plenum Press.

25. Miller, T. 1978. The insect neuromuscular system as a site of insecticide action. *In*: "Pesticides and Venom Neurotoxicity," Ed. by D. L. Shankland, R. F. Flattum, R. M. Hollingworth and T. Smyth, Jr., New York, Plenum Publ. Co., pp. 95-111.

26. Olsen, R. W., R. Ticku, and T. Miller. 1978. Dihydropicrotoxinin binding to crayfish muscle sites possibly related to GABA receptor-iontophores. Molec. Pharmacol. 14: 391-402.

27. Umetsu, N., M. A. H. Fahmy, and T. R. Fukuto. 1979. Metabolism of 2,3-dihydro-2,2-dimethyl-7-benzofuranyl (di-n-butylaminosulfenyl)(methyl)carbamate and 2,3-dihydro-2,2-dimethyl-7-benzofuranyl (morpholinsulfenyl)(methyl)carbamate in cotton and corn plants. Pestic. Biochem. Physiol. 10: 104-119.

28. Abu-El-Haj, S., M. A. H. Fahmy, and T. R. Fukuto. 1979. Insecticidal activity of DDT analogs. J. Agric. Food Chem. 27: 258-261.

29. Mallipudi, N. M., and T. R. Fukuto. 1979. Toxicity of *N*-sulfenylated derivatives of insecticidal methylcarbamate esters to the honey bee. J. Agric. Food Chem. 27: 261-265.

30. Mallipudi, N. M., N. Umetsu, R. F. Toia, R. E. Talcott, and T. R. Fukuto. 1979. Toxicity of *O*,*O*,*S*-trimethyl and triethyl phosphorothioate to the rat. J. Agric. Food Chem. 27: 463-466.

31. Talcott, R. E., N. M. Mallipudi, N. Umetsu, and T. R. Fukuto. 1979. Inactivation of esterases by impurities isolated from technical malathion. Toxicol. Appl. Pharmacol. 49: 107-112.

32. Umetsu, N., R. F. Toia, N. M. Mallipudi, and T. R. Fukuto. 1979. A novel antagonistic effect to the toxicity in the rat of *O*,*O*,*S*-trimethyl phosphorothioate by its phosphorothionate isomer. J. Agric. Food Chem. 27: 1423-1425.

33. Talcott, R. E., N. M. Mallipudi, and T. R. Fukuto. 1979. Malathion carboxylesterase titer and its relationship to malathion toxicity. Toxicol. Appl. Pharmacol. 50: 501-504.

34. Hollingshaus, J. G., S. Abu-El-Haj, and T. R. Fukuto. 1979. The neurotoxicity of *O*-alkyl *O*-aryl phenylphosphonothioate analogs related to leptophos. J. Agric. Food Chem. 27: 1197-1201.

35. Miller, T. 1979. Mode of action of insecticides: Insights gained from neurophysiological preparations of intact or dissected insects. *In*: Neurotoxicology of Insecticides and Pheromones, ed. by Toshio Narahashi, pp. 79-96. New York: Plenum Press.

36. Miller, T., M. Maynard, and J. M. Kennedy. 1979. Structure-activity studies of picrotoxinin. Pestic. Biochem. Physiol. 10: 128-136.

37. Collins, C., J. M. Kennedy, and T. A. Miller. 1979. Sublethal poisoning: A comparison of behavior and histological changes in house fly CNS. Pestic. Biochem. Physiol. 11: 135-158.

38. Adams, M. E., and T. A. Miller. 1979. Site of action of pyrethroids: Repetitive "backfiring" in flight motor units of house fly. Pestic. Biochem. Physiol. 11: 218-231.

39. Talcott, R. E., H. Denk, and N. M. Mallipudi. 1979. Malathion carboxylesterase activity in human liver and its inactivation by isomalathion. Toxicol. Appl. Pharmacol. 49: 373-376.

40. Irving, S. N. and T. A. Miller. 1979. Ionic differences in 'fast' and 'slow' neuromuscular transmission in body wall muscles of *Musca* larvae. J. Comp. Physiol. 135: 291-298.

41. Irving, S. N., and T. A. Miller. 1979. Glutamate and aspartate as possible transmitters of the 'fast' and 'slow' neuromuscular junctions of body wall muscles of *Musca* larvae. J. Comp. Physiol. 135: 299-314.

42. Miller, T. A., J. M. Kennedy, and C. Collins. 1979. CNS insensitivity to pyrethroids in the resistant *kdr* strain of house flies. Pestic. Biochem. Physiol. 12: 224-230.

43. Mallipudi, N. M., and T. R. Fukuto. 1979. Penetration and metabolism of 2-isopropoxyphenyl N-methyl-N-(2-methyl-4-*t*-butylphenylsulfenyl)carbamate in the house fly and honey bee. J. Pestic. Biochem. Physiol. 12: 55-67.

44. Lee, P. W., R. Allahyari, and T. R. Fukuto. 1980. Absorption and metabolism of the chiral isomers of fonofos in the corn and cotton plant. J. Environ. Science Hlth. B15(1): 25-37.

45. Umetsu, N., E. Kuwano, and T. R. Fukuto. 1980. Nature of N-S bond cleavage of 2,3-dihydro-2,2-dimethyl-7-benzofuranyl (di-n-butylaminosulfenyl)(methyl)carbamate. J. Environ. Science Hlth. B15(1): 1-23.

46. Toia, R. F., R. B. March, N. Umetsu, N. M. Mallipudi, R. Allahyari, and T. R. Fukuto. 1980. Identification and toxicological evaluation of impurities in technical malathion and fenthion. J. Agric. Food Chem. 28: 599-604.

47. Allahyari, R., J. G. Hollingshaus, R. L. Lapp, E. Timm, R. A. Jacobson and T. R. Fukuto. 1980. Resolution, absolute configuration, and delayed toxicity of the chiral isomers of *O*-aryl *O*-methyl phenylphosphonothioate analogs related to leptophos. J. Agric. Food Chem. 28: 594-599.

48. Miller, T. A. 1980. Insect visceral and cardiac nerve-muscle systems. *In*: Insect Neurobiology and Pesticide Action, pp. 145-152, London: Society of Chemical Industry.

49. Irving, S. N. and T. Miller. 1980. L-Glutamate and asparate as possible transmitters at 'fast' and 'slow' neuromuscular junctions in body-wall muscle of *Musca*larvae. *In*: Insect Neurobiology and Pesticide Action, pp. 227-234, London: Society of Chemical Industry.

50. Adams, M. E. and T. A. Miller. 1980. Neurophysiological correlates of pyrethorid and DDT-type insecticide poisoning in the house fly, *Musca domestica* L. *In*: Insect Neurobiology and Pesticide Action, pp. 431-440, London: Society of Chemical Industry.

51. Mallipudi, N. M., R. E. Talcott, A. Ketterman, and T. R. Fukuto. 1980. Properties and inhibition of rat malathion carboxylesterases. J. Toxicol. Environ. Hlth. 6: 587-598.

52. Collins, C., J. M. Kennedy, M. A. H. Fahmy, and T. A. Miller. 1980. Mode of action of sulfenylated carbamates: Rapid conversion of *N*,*N*'-thiodicarbamates to parent carbamate measured by neurophysiological bioassay. Pestic. Biochem. Physiol. 13: 158-163.

53. Adams, M. E. and T. A. Miller. 1980. Neural and behavioral correlates of pyrethroid and DDT-type poisoning in the house fly, *Musca domestica* L. Pestic. Biochem. Physiol. 13: 137-147.

54. Clay, V. E., J. P. Martin and T. R. Fukuto. 1980. Degradation of 2,3-dihydro-2,2-dimethyl-7-benzofuranyl (di-*n*-butylaminosulfenyl)(methyl)carbamate in Cosad sandy loam. J. Agric. Food Chem. 28: 1122-1129.

55. Clay, V. E., J. P. Martin, and T. R. Fukuto. 1980. Degradation of 2,3-dihydro-2,2-dimethyl-7-benzofuranyl)(morpholinsulfenyl)-(methyl)carbamate in Cosad sandy loam. J. Agric. Food Chem. 28: 1129-1130.

56. Kinoshita, Y., and T. R. Fukuto. 1980. Insecticidal properties of *N*-sulfonyl derivatives of propoxur and carbofuran. J. Agric. Food Chem. 28: 1325-1327.

57. Umetsu, N., N. M. Mallipudi, R. F. Toia, R. B. March and T. R. Fukuto. 1981. Toxicological properties of phosphorothioate and related esters present as impurities in technical organophosphorus insecticides. J. Toxicol. Environ. Hlth. 7: 481-497.

58. Fukuto, T. R. and N. M. Mallipudi. Suppression of metabolic resistance thorugh chemical structure modification, *In*: Pest Resistance to Pesticides: Challenges and Prospects, G. P. Georghiou and T. Saito, eds., New York, Plenum Press. (in press)

59. Miller, T., V. Salgado, and S. N. Irving. 1980. The *kdr* factor in pyrethroid resistance. *In*: Pest Resistance to Pesticides: Challenges and Prospects, G. P. Georghou and T. Saito, eds., New York: Plenum Press. (in press)

60. Mallipudi, N. M., and T. R. Fukuto. Characterization of bound phenthoate residues in citrus. Arch. Environ. Contamin. Toxicol. (in press)

61. Hollingshaus, J. G., T. Nishioka, R. B. March, and T. R. Fukuto. The effect of impurities on the delayed neurotoxicity of *O*-(4-bromo-2,5-dichlorophenyl) *O*-ethyl phenylphosphonothioate administered orally to hens. J. Agric. Food Chem. (in press)

62. Hollingshaus, J. G., D. Armstrong, R. F. Toia, L. McCloud, and T. R. Fukuto. Delayed toxicity and delayed neurotoxicity of phosphoro- and phosphonothioate esters. J. Toxicol. Environ. Hlth. (in press)

63. Umetsu, N., T. Nishioka, and T. R. Fukuto. Acid catalyzed alteration of 2,3-dihydro-2,2-dimethyl-benzofuranyl-7 (di-*n*-butylaminosulfenyl)(methyl)carbamate via N-S bond cleavage. I. Formation of polysulfide derivatives. J. Agric. Food Chem. (in press)

64. Umetsu, N., T. Nishioka, and T. R. Fukuto. Acid catalyzed alteration of DBSC [2,3-dihydro-],]-dimethyl-benzofuranyl-7 (di-n-butylaminosulfenyl)(methyl)carbamate via N-S bond cleavage. II. Separation and identification of polysulfide derivatives. J. Agric. Food Chem. (submitted)

65. Nishioka, T., N. Umetsu, and T. R. Fukuto. Side-chain group metabolism of the dibutylaminosulfenyl derivative of carbofuran in the cotton plant. Pestic. Biochem. Physiol. (submitted)